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# OXIDATION OF AMMONIA AND UREA (HETEROTROPHIC NITRIFICATION) BY STREPTOMYCES NITRIFICANS [1]

ALBERT SCHATZ AND R. RAM MOHAN
*National Agricultural College, Farm School P. O.,*
*Bucks County, Pennsylvania*

TWO FIGURES

Since the classical work of Winogradsky (1890, 1891), nitrification has generally been considered to be restricted to the obligately chemoautotrophic *Nitrobacterieae* despite reports which indicate that some heterotrophic organisms may also nitrify. Heterotrophic bacteria (Devignat, '52; Fisher et al., '52; Hutton and Zobel, '53; Karlson, '54), actinomycetes (Rebstock et al., '49; Isenberg et al., '54; Hirata et al., '54), and molds (Bush et al., '51; Schmidt, '54) produce nitrite, nitrate, and organic nitro compounds from ammonia, urea, guanidine, carbamates, amino acids, proteins, etc. In addition, the production of hydroxylamine (Steinberg, '39) and oximes (Virtanen et al., '49) from reduced nitrogen may not be uncommon among heterotrophs. Aside from these kinds of oxidative nitrification, many heterotrophic organisms hydrolyze oximes to nitrite (Quastel and Scholefield, '49; Jensen, '51; Quastel et al., '52). The *Nitrobacterieae* do not carry out this reaction which, in contrast to the oxidation of ammonia, is probably not exergonic (Quastel and Scholefield, '49; Jensen, '50).

Oxime hydrolysis occurs rapidly and the organisms which nitrify in this manner are easily cultured. Consequently, a study of this process has recently been proposed as a convenient approach to the general problem of nitrification by the

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obligate chemoautotrophs (Lees et al., '54). This indeed appears advantageous since *Nitrobacterieae* are generally difficult to work with (Meiklejohn, '54). For this reason many investigations with these bacteria have understandably been carried out on impure cultures. Nevertheless, much valuable information has been accumulated (Quastel and Scholefield, '51). However, relatively little is known about the intermediary biochemistry of nitrification as compared with certain other areas of microbial metabolism such as glycolysis, terminal respiration, transamination, bacterial photosynthesis, etc. In this respect, the nitrification by *Streptomyces nitrificans,* which has been studied since 1952 (Isenberg et al., '52), may offer a more direct approach since conditions for the rapid oxidation of ammonia and other nitrogenous substrates by non-proliferating cells have finally been defined. It is thus now possible, as here reported, to follow the nitrification reaction manometrically within a two to three hour interval.

## METHODS

*S. nitrificans* was cultured in a synthetic medium containing 0.2% urethan as the sole source of nitrogen, carbon, and energy (Schatz et al., '54). The production of cell material, the method of preparing homogenates, and conditions for Warburg experiments have already been reported (Mohan et al., in press). Experiments were carried out on the day of harvest since the stability of the nitrifying mechanism during storage is unknown. Nitrite was determined by diazotization and the resulting red color was estimated at 530 mμ in a Beckman quartz spectrophotometer. Stock substrate solutions and cell homogenates were ascertained to be free of nitrite at the time they were used. The quotients for rates of respiration have all been calculated as $Q_{O_2}(N) = \mu l\ O_2$ consumed per milligram cell nitrogen per hour.

## RESULTS

The results presented in figure 1 are typical of those that have been obtained in repeated experiments. The curves show that $O_2$ uptake with $(NH_4)_2CO_3$ began immediately after tipping and continued at an increasing rate. By the second hour gas consumption with this substrate was appreciably greater than the $O_2$ uptake of autorespiration. Nevertheless, the experiment was continued 9 hours to see whether breaks in the $(NH_4)_2CO_3$ curves would appear. The oxidation of urea which began after a lag period of about $3\frac{1}{6}$ hours occurred at a greater rate than for $(NH_4)_2CO_3$. Ethanol was included for comparison with $(NH_4)_2CO_3$ and urea, since this alcohol is

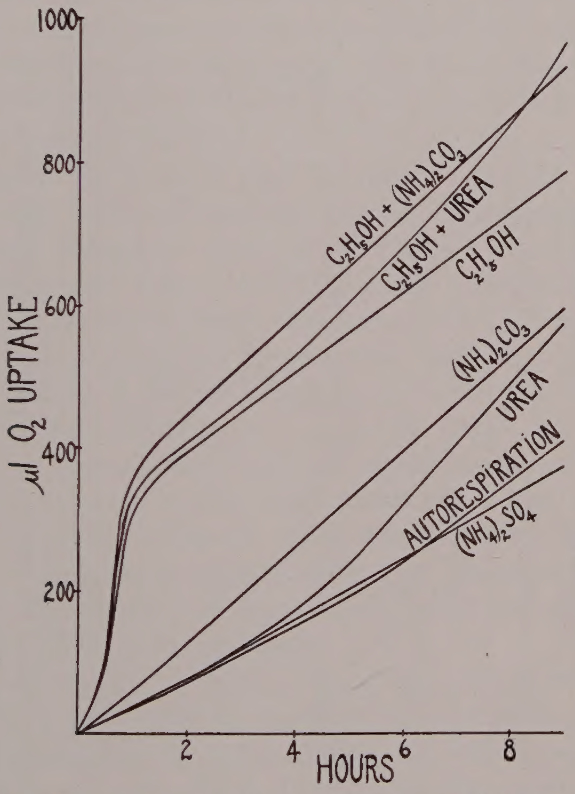

Fig. 1 Autorespiration and substrate oxidation by *Streptomyces nitrificans.* Contents per vessel: 2.1 ml homogenized cell material equivalent to 1.4 mg nitrogen suspended in 0.024 M phosphate buffer containing trace metals as used by Mohan et al. (in press). pH 7.0. 10.0 μM ethanol and 30.0 μM other substrates. KOH in center well. 30°C.

TABLE 1

*Respiratory activity of Streptomyces nitrificans during periods of substrate oxidation*

| SUBSTRATE | | TIME INTERVAL, HOURS | CORRECTED FOR AUTORESPIRATION | | | CORRECTED FOR ETHANOL OXIDATION[1] | | | CORRECTED FOR OXIDATION OF $(NH_4)_2CO_3$ AND UREA[1] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | µl $O_2$ uptake | $Q_{O_2}$ (N) | Per cent substrate oxidized[2] | µl $O_2$ uptake | $Q_{O_2}$ (N) | Per cent substrate oxidized[2] | µl $O_2$ uptake | $Q_{O_2}$ (N) | Per cent substrate oxidized |
| | µM | | | | | | | | | | |
| $(NH_4)_2CO_3$ | 30.0 | 0 to 9 | 187 | 15 | 9 | | | | | | |
| Urea | 30.0 | $3\frac{1}{6}$ to 9 | 166 | 20 | 8 | | | | | | |
| Ethanol | 10.0 | 0 to $\frac{5}{6}$ | 274 | 230 | 41 | | | | | | |
| Ethanol | 10.0 | 0 to $\frac{5}{6}$ | 276[3] | 240[3] | | | | | 250[5] | 210[5] | 37[5] |
| plus | | | | | | | | | | | |
| $(NH_4)_2CO_3$ | 30.0 | $\frac{5}{6}$ to 9 | | | | 151[4] | 12[4] | 8[4] | | | |
| Ethanol | 10.0 | 0 to $\frac{5}{6}$ | 293[3] | 250[3] | | | | | 301[5] | 260[5] | 45[5] |
| plus | | | | | | | | | | | |
| Urea | 30.0 | $3\frac{1}{6}$ to 9 | | | | 165[4] | 20[4] | 8[4] | | | |
| None (autorespiration) | | 0 to 9 | 416 | 33 | | | | | | | |

[1] Including autorespiration.

[2] Computed on the basis of 2.016 ml $O_2$ required for complete oxidation of $(NH_4)_2CO_3$ and urea to nitrite.

[3] Ethanol plus $(NH_4)_2CO_3$ and ethanol plus urea.

[4] $(NH_4)_2CO_3$ and urea.

[5] Ethanol alone.

rapidly oxidized by urethan-grown *S. nitrificans* (Mohan et al., in press). According to the data in table 1, the respective rates of oxidation of ammonia ($Q_{O_2} = 15$), urea ($Q_{O_2} = 20$), and ethanol ($Q_{O_2} = 230$) are approximately one-half, two-thirds, and 7 times the rate of autorespiration ($Q_{O_2} = 33$). For urea and ethanol, these quotients are significantly greater when calculated over the intervals during which these substrates were actually being oxidized than for the entire 9 hour period of the experiment (table 2).

The curves in figure 1 and the data in table 1 show that ethanol was oxidized rapidly but at increasingly greater rates when supplied alone ($Q_{O_2} = 230$), with $(NH_4)_2CO_3$ ($Q_{O_2} = 240$), and with urea ($Q_{O_2} = 250$). Since the amount of alcohol respired in these vessels was 41, 37, and 45%, respectively, the presence of ammonia and urea as readily available nitrogen did not seriously alter the extent of oxidative assimilation of the alcohol. After the break in the ethanol-$(NH_4)_2CO_3$ curve, the rate of oxidation of $(NH_4)_2CO_3$ ($Q_{O_2} = 12$) is close to the value obtained in the absence of alcohol ($Q_{O_2} = 15$). The corresponding quotients for urea are both $Q_{O_2} = 20$. These results show that the oxidation product(s) of alcohol did not appreciably affect the rates of respiration of $(NH_4)_2CO_3$ and urea.

As shown by the Q values in table 1, urea was oxidized more rapidly than $(NH_4)_2CO_3$ when each of these compounds was provided alone or with alcohol. *S. nitrificans* required $8\frac{1}{6}$ and 9 hours to oxidize 8 and 9%, respectively, of the $(NH_4)_2CO_3$ depending on whether ethanol had also been added or not. But in $5\frac{5}{6}$ hours, 8% of the urea was utilized with and without alcohol as a supplementary substrate. It is not yet known whether this greater rate of oxidation of urea as compared with $(NH_4)_2CO_3$ is generally characteristic of *S. nitrificans* or is specifically a function of cell material grown on urethan. One might assume that urea would first be hydrolyzed and the resulting ammonia then oxidized. If this were so, urea should be nitrified at about the same rate as $(NH_4)_2CO_3$

TABLE 2

*Nitrification and oxygen uptake by Streptomyces nitrificans for the entire 9 hour experiment*

| SUBSTRATE | | CORRECTED FOR AUTORESPIRATION | | CORRECTED FOR ETHANOL OXIDATION [1] | | CORRECTED FOR OXIDATION OF $(NH_4)_2CO_3$ AND UREA [1] | | NITRIFICATION OF $(NH_4)_2CO_3$ AND UREA | |
|---|---|---|---|---|---|---|---|---|---|
| | $\mu M$ | $\mu l\ O_2$ uptake | $Q_{O_2}$ (N) | $\mu l\ O_2$ uptake | $Q_{O_2}$ (N) | $\mu l\ O_2$ uptake | $Q_{O_2}$ (N) | mg nitrite formed per vessel (2.1 ml) | Per cent oxidized [2] |
| $(NH_4)_2CO_3$ | 30.0 | 187 | 15 | | | | | 0.248 | 9 |
| Urea | 30.0 | 164 | 13 | | | | | 0.284 | 10 |
| Ethanol | 10.0 | 379 | 30 | | | | | 0.013 | $< 0.5$ |
| Ethanol<br>plus<br>$(NH_4)_2CO_3$ | 10.0<br><br>30.0 | 532 | 42 | 153 | 12 | 345 | 27 | 0.193 | 7 |
| Ethanol<br>plus<br>Urea | 10.0<br><br>30.0 | 564 | 45 | 185 | 15 | 400 | 32 | 0.221 | 8 |
| $(NH_4)_2SO_4$ | 30.0 | — 36 [3] | — 3 [3] | | | | | 0.013 | $< 0.5$ |
| None (autorespiration) | | 416 | 33 | | | | | 0.013 | $< 0.5$ |

[1] Including autorespiration.

[2] Computed on the basis of complete oxidation of $(NH_4)_2CO_3$ and urea to 2.82 mg nitrite.

[3] Negative values indicate partial inhibition of autorespiration.

because *S. nitrificans* possesses a highly active urease. On the other hand, ammonia may be combined with some organic compound before being oxidized, in which case urea might be closer to the ''active'' intermediate. Such a pathway is suggested by oxime nitrification (Jensen, '51), but the adaptation period required by urea does not support this kind of mechanism for *S. nitrificans.* It is paradoxical that the compound which is nitrified at the more rapid rate requires an adaptation period while oxidation of the more slowly utilized substrate commences at once.

The failure of *S. nitrificans* to oxidize $(NH_4)_2SO_4$ may be due to unfavorable pH. This is indicated by the fact that autotrophic bacterial nitrification occurs best in the alkaline range (Lees, '54) and by the behavior of the specific ammonium dehydrogenase of *Bacillus subtilis* (Klausmeier and Bard, '54). In an alkaline medium, this enzyme, which is mediated by di- or triphosphopyridine nucleotide, converts $NH_4OH$ to $H_2NOH$, but it does not act on $NH_4Cl$ or $(NH_4)_2SO_4$. Under the experimental conditions employed with *S. nitrificans,* both urea and $(NH_4)_2CO_3$ would tend to alkalize the medium after absorption of $CO_2$ by KOH in the center well. The acidic $(NH_4)_2SO_4$, on the other hand, would tend to lower the pH.

The increased rate of $O_2$ uptake with $(NH_4)_2CO_3$ and urea is not itself conclusive evidence of nitrification. Conceivably, the presence of these nitrogenous substrates might in some way stimulate endogenous gas uptake. However, the analytical results presented in table 2 show a 15- to 22-fold increase in nitrite content of those vessels supplied with $(NH_4)_2CO_3$ and urea. The low nitrite in the endogenous vessel and in the flasks containing ethanol and $(NH_4)_2SO_4$ may be due to oxidation of nitrogenous reserve food material since *S. nitrificans* produces nitrite during growth on a wide variety of organic nitrogen sources. This low level of endogenous nitrite formation has been observed in some experiments but in others the autorespiration flasks contained no demonstrable nitrite.

On the basis of $O_2$ uptake (table 1), *S. nitrificans* oxidized 9 and 8% of the $(NH_4)_2CO_3$ in the absence and presence of alcohol, respectively, as compared with the 9 and 7% values calculated from the nitrite analyses (table 2). For urea, the corresponding values are 8 and 8% as compared with 10 and 8%. In view of the experimental error inherent in the methods employed, the correlation between total $O_2$ uptake and nitrite formed is considered to be quantitatively satisfactory and is taken as evidence of actual nitrification by *S. nitrificans.*

## DISCUSSION

The ability to reduce nitrate to ammonia and organic compounds is widely distributed among microflora, higher plants, and animals. These organisms also reduce nitrite, to which nitrate is first converted. But in contrast to this, the oxidation of ammonia to nitrite and of nitrite to nitrate appears to be carried out primarily by the two specific groups of bacteria known as the *Nitrobacterieae.* Why ammonia oxidation does not occur more widely among the numerous heterotrophs capable of assimilating nitrate is not understood. These organisms enzymatically reduce nitrate to nitrite and nitrite to ammonia, amino acids, etc. They would, therefore, be expected *a priori* to effect the reverse reactions. With ammonia available, nitrification might provide heterotrophs and even photosynthetic organisms (e.g. algae in the dark) with an additional energy supply which may be of some survival advantage. Yet ammonia and nitrite appear to be the only inorganic chemoautotrophic energy substrates which are not oxidized by numerous heterotrophs.

Whether ammonia oxidation by the strictly heterotrophic *S. nitrificans* is carried out in a manner similar to nitrification by the obligately autotrophic bacteria remains to be determined. It is also not known whether this actinomycete actually utilizes the energy released by oxidation of ammonia. From the point of view of comparative biochemistry, information on the intermediary metabolism of the nitrification effected by

*S. nitrificans* would be of interest if the pathway is different from that of the autotrophic bacteria. Notwithstanding, a knowledge of the mechanism of nitrification by *S. nitrificans* may possibly be helpful in handling and understanding the *Nitrobacterieae.* Whereas soil profusion tests (Quastel and Scholefield, '51) indicate that autotrophic bacterial nitrification is carried out by actively multiplying cells, nitrification by *S. nitrificans* is effected both in growing cultures as well as by non-proliferating cells. This suggests that the actinomycete may oxidize ammonia in a different manner from the *Nitrobacterieae.* On the other hand, *S. nitrificans* differentiates between ammonia and nitrite as respiration substrates, as do the nitrifying bacteria. In Warburg experiments nitrite is not oxidized although the organism grows equally well with ammonia, nitrite, nitrate and urea as sources of nitrogen.

Carbamate esters inhibit multiplication, respiration, and ammonia oxidation by *Nitrobacterieae* in soil, perfusion experiments, and pure culture studies (Meyerhof, '16, '17; Lees and Quastel, '46; Quastel and Scholefield, '49, '51). Mixtures of urethan and ammonia are nitrified in the soil only after microbiological destruction of the former has taken place (Quastel and Scholefield, '53). But *S. nitrificans* grows on carbamates and also respires and nitrifies these compounds. That a urethan-utilizing organism nitrifies $(NH_4)_2CO_3$, urea, and carbamates is of interest because of the relationships of these compounds as shown in figure 2. Urethan may be hydrolyzed to ethanol and carbamic acid. The latter is unstable and spontaneously decomposes to $CO_2$ and $NH_3$. These products react with $H_2O$ to form $(NH_4)_2CO_3$ which exists in aqueous solution as an equilibrium mixture of ammonium carbamate and ammonium carbonate. Ammonium carbamate, in turn, can be dehydrated to urea (Karrer, '46).

It is a pleasure to acknowledge the contribution of Dr. Seymour H. Hutner of the Haskins Laboratories, New York City, to the work which is here reported. In 1946, Doctor Hutner suggested these studies which began with the microbial metabolism of carbamates. He collaborated with one of us

(A. S.) on the enrichment culture isolation and early experiments on *S. nitrificans,* and also speculated at that time on the possibility of heterotrophic nitrification by a urethan-utilizing organism.

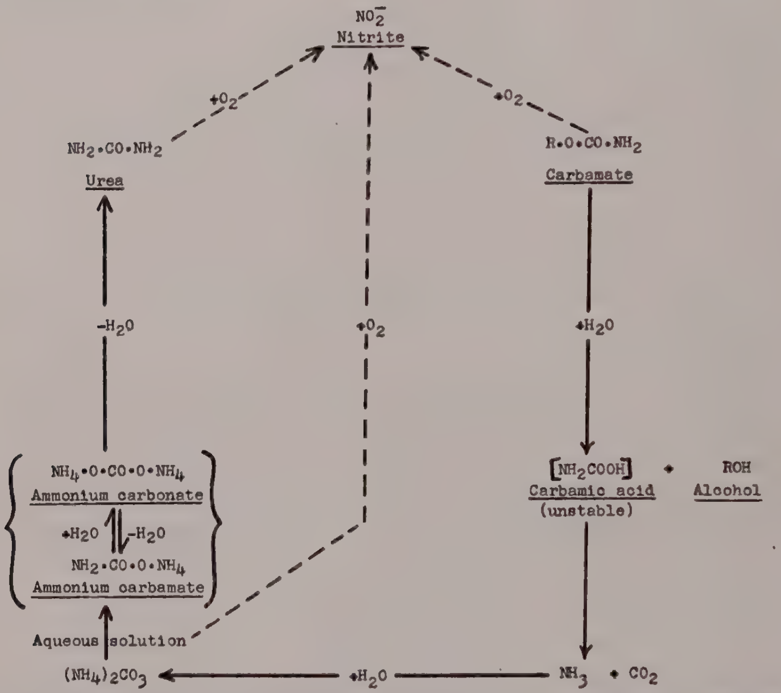

Fig. 2 Relationships among compounds nitrified by *S. nitrificans.* Ammonium carbonate and ammonium carbamate are indicated as an equilibrium mixture in aqueous solution. Nitrification is shown by a broken line.

## SUMMARY

*S. nitrificans* may be a useful tool for studying the intermediary metabolism of nitrification divorced from cell proliferation. Urethan-grown cells of this organism are easy to obtain and appear to be ideally suitable for manometric experiments in which the oxidation of $(NH_4)_2CO_3$ can be conveniently studied within intervals as short as two hours. Nitrification of $(NH_4)_2CO_3$ and urea was demonstrated by $O_2$ uptake as well as by quantitative determination of nitrite. Both methods showed that during a 9 hour experiment ap-

proximately 9% of each substrate was oxidized when supplied alone or with alcohol. While utilization of $(NH_4)_2CO_3$ commenced at once, $O_2$ uptake with urea occurred only after a $3\frac{1}{6}$ hour adaptation period. Nitrite and hydroxylamine were not oxidized. Nitrification reactions by *S. nitrificans* and by the obligately autotrophic *Nitrobacterieae* are compared. The interrelationships between ammonia, urea, and carbamates are discussed.

## ADDENDUM

After this manuscript had been submitted, additional experiments have shown that pyruvic oxime (10.0 to 90.0 µM per vessel) causes a slight increase in oxygen uptake and releases small amounts of $H_2NOH$ in Warburg experiments with homogenized *S. nitrificans* grown on urethan. However, $H_2NOH$ (1.0 to 100.0 µM per vessel) was not respired. Spectrophotometric studies with cell-free preparations (obtained by grinding damp mycelium and subsequent centrifugation) established the presence of a heat-labile ammonium dehydrogenase that is DPN- or TPN-linked. The oxidized coenzymes were reduced with $NH_4OH$ and then reoxidized upon addition of acetaldehyde. The reverse reaction was obtained with reduced coenzymes and $H_2NOH$.

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# THE RESPIRATORY METABOLISM OF FROGS AS RELATED TO SEASON[1]

PAUL O. FROMM[2] AND ROBERT E. JOHNSON
*College of Liberal Arts and Sciences, Department of Physiology,*
*University of Illinois, Urbana*

FOUR FIGURES

It has been recognized for years that frog tissues show seasonal differences in their physiological behavior, and it is customary to distinguish between summer and winter frogs. Many measurements of the respiratory metabolism of frogs have been made in past years. A survey of the literature has shown that there is considerable variability in the total metabolism between the different species of frogs as well as large individual variations within a single species. Often the variations within a single species are far greater than the normal variability found in individuals among the warm-blooded animals.

A summary of observations on frog metabolism from 1849 to 1954 is given in table 1. The data suggest that there are variations in the oxygen consumption associated with the seasons and also within a single season. For the most part, measurements were made by investigators during a single season and in only a few isolated cases were appreciable amounts of data obtained during all seasons of the year. Although there are irregularities, maximal values for the oxygen consumption of a frog occurred generally during

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[1] Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Physiology in the Graduate College of the University of Illinois, 1954.

[2] Present address: Department of Physiology and Pharmacology, Michigan State University, East Lansing, Michigan.

TABLE 1

*Literature on respiratory metabolism of frogs*

| SPECIES | TIME OF YEAR | $O_2$ CONS.[1] | $CO_2$ PROD.[1] | R.Q. | TEMP. OF ANIMALS, °C. | REF. NO. |
|---|---|---|---|---|---|---|
| *R. esculenta* | Jan. | 9.70 | 11.80 | 1.22 | 25.0 | 16 |
| *R. esculenta* | Feb. | 9.40 | 13.50 | 1.47 | 24.5 | 16 |
| *R. esculenta* | Feb. | 6.10 | 4.70 | 0.77 | ? | 11 |
| *R. esculenta* | March | 8.25 | 6.58 | 0.75 | 10–20 | 4 |
| *R. esculenta* | March | 21.20 | 16.80 | 0.88 | 19.0 | 4 |
| *R. esculenta* | March | 7.90 | 6.00 | 0.76 | ? | 11 |
| *R. esculenta* | April | 12.00 | 12.40 | 1.07 | 20–25 | 16 |
| *R. esculenta* | April | 13.60 | 11.83 | 0.87 | ? | 11 |
| *R. esculenta* | June | 5.05 | 4.80 | 0.95 | 17.7 | 20 |
| *R. esculenta* | June | ? | 4.17 | ? | 17.0 | 28 |
| *R. esculenta* | June | 11.60 | 7.98 | 0.68 | 20.0 | 4 |
| *R. esculenta* | June | 15.20 | 13.70 | 0.91 | 20–21 | 16 |
| *R. esculenta* | July | 7.40 | 5.25 | 0.71 | 22–27 | 6, 7 |
| *R. esculenta* | July | 1.63 | 1.16 | 0.71 | 10.0 | 6, 7 |
| *R. esculenta* | Sept.[2] | 4.42 | 3.08 | 0.69 | 15–19 | 24 |
| *R. esculenta* | Oct.[2] | 7.34 | 5.77 | 0.78 | 15–19 | 24 |
| *R. esculenta* | Dec. | 8.70 | 9.90 | 1.15 | 25.0 | 16 |
| *R. esculenta* | winter | 5.43 | 4.45 | 0.82 | 20.0 | 30, 31 |
| *R. esculenta* | ? | ? | 6.51 | ? | 19.0 | 2 |
| *R. temporaria* | Jan. | 6.89 | 6.68 | 0.97 | 24.7 | 5 |
| *R. temporaria* | Feb. | 9.94 | 9.57 | 1.00 | 24.7 | 5 |
| *R. temporaria* | March | 12.70 | 11.02 | 0.87 | 24.7 | 5 |
| *R. temporaria* | April | 20.00 | 17.40 | 0.85 | 19.3 | 4 |
| *R. temporaria* | April | 17.30 | 16.86 | 0.97 | 24.7 | 5 |
| *R. temporaria* | May | 15.69 | 15.48 | 0.98 | 24.7 | 5 |
| *R. temporaria* | July | 6.99 | 4.60 | 0.66 | 19–25 | 15[3] |
| *R. temporaria* | Oct. | 11.37 | 11.96 | 1.05 | 24.7 | 5 |
| *R. temporaria* | Nov. | 2.69 | 1.83 | 0.72 | 24.7 | 5 |
| *R. temporaria* | winter | ? | 1.93 | ? | 13.0 | 22 |
| *R. temporaria* | winter | 6.56 | 5.58 | 0.85 | 22.5 | 30, 31 |
| *R. temporaria* | ? | ? | 25.65 | ? | 23.0 | 19 |
| *R. temporaria* | ? | ? | 18.01 | ? | 17.0 | 23 |
| *R. fusca* | April | 19.40 | 21.00 | 1.09 | 20.0 | 16 |
| *R. fusca* | May | 18.20 | 21.30 | 1.22 | 20.0 | 16 |
| *R. fusca* | Oct. | 10.70 | 12.40 | 1.15 | 20.0 | 16 |
| *R. fusca* |  | 7.78 | ? | ? | 26.3 | 14 |
| *R. pipiens* | March | 4.40 | ? | ? | 22.0 | 20 |
| *R. pipiens* | summer | 16.60 | 11.10 | 0.70 | 25–28 | 18 |
| *R. pipiens* | winter | ? | ? | 0.74 | 19.5 | 22 |
| *R. viridis* | Oct. | 0.57 | 0.40 | 0.70 | 5.0 | 6, 7 |
| *R. viridis* | Dec. | 0.84 | 0.59 | 0.70 | 5.0 | 6, 7 |
| *R. fusca and R. esculenta* | June-Sept. | 6.19 | 4.83 | 0.77 | 19–24 | 1 |
|  | Oct.-Mar. | 2.71 | 2.72 | 1.00 | 7–16.5 | 1 |
| *Sp. not given* | winter | ? | ? | 0.73 | ? | 29 |
| *Sp. not given* |  | 13.00 | ? | ? | 23.0 | 17 |

[1] Oxygen consumption and carbon dioxide production reduced to standard temperature and pressure. Data presented as ml/100 gm/hr., wet weight.

[2] Krogh ('04) believed these animals to have been measured in the fall.

[3] Kayser ('40) found no difference between summer and winter.

the spring associated with the spawning season and minimal values occurred during the winter. Carbon dioxide production followed a somewhat similar pattern during the various seasons of the year.

R.Q. values reported for frogs have ranged from below 0.7 to higher than 1.4. There are indications (Athanasiu, '00; Krogh, '04) that during the summer the R.Q. is lower than during the winter. However, other data (Dolk and Postma, '27) show that just the reverse is true. Finally, Kayser ('40) observed no significant differences between the R.Q. for summer and winter frogs.

Frogs are active cold-blooded animals and activity during any experimental period prevents, to a large degree, the collection of metabolic data valid for comparative purposes. It is most probable that the activity of the experimental animals in the many observations cited has given rise to some of the variability in the results. Differences in the nutritional status of the animals, conditions under which the animals were stored prior to experiments, and collection of data at different temperatures may also have given rise to some irregularities. A variety of calorimeters was used by the various investigators and other methodological differences may also have contributed to discrepancies.

The present experiment was designed to measure the respiratory metabolism of intact frogs *(Rana pipiens)* during all months of the year and to establish, if possible, how much variation occurs in the oxygen consumption, carbon dioxide production and R.Q. during the various seasons. To prevent any effect by the activity of the frogs, the experimental animals were completely immobilized with d-tubocurarine chloride (Abbott). In addition, the experiment tested the effects of storage at 4°C. for prolonged periods of time and of feeding.

## MATERIALS AND METHODS

*Rana pipiens* were obtained from a commercial dealer in Wisconsin, their previous history being unknown. On arrival they were divided into 4 groups. Two groups, one fed and one unfed, were kept for at least two weeks in an air conditioned room at 22°–28°C. The room was well lighted with fluorescent lights which remained on at all times. The frogs were kept in a stainless steel tank with tap water continually dripping into the tank through a sprinkler system. Approximately 8 frogs were kept in each group at all times and the animals remained quite healthy, with less than 2% loss from the disease "red leg." Mortality from other causes was also low. In all, 5 fed and three unfed frogs survived the year.

The other two groups, one fed and one unfed, were placed for at least two weeks in a refrigerator and kept at an average temperature of 4°C. While they remained in the refrigerator they were not exposed to any light. Approximately 20% of these animals developed "red leg," most cases occurring during September, November, December and May. When the frogs contracted the disease they were sacrificed and replaced with healthy frogs. Mortality from other cases was high. No frog survived in the refrigerator for longer than 6 months.

Every 5 days each animal of the "fed groups" received 0.5 ml of an homogenate of tuna fish (El Rawi and Geiger, '52). The mixture was injected into the stomach from a tuberculin syringe through a small plastic tube. The nutrients provided in 0.5 ml were: calories 367; protein 0.085 gm; fat 0.003 gm; carbohydrate none.

The frogs were immobilized by injection into the dorsal lymph sac of 0.10 ml d-tubocurarine chloride (Abbott), an amount sufficient for complete immobility for at least 4 hours. Recovery was uneventful. Judging by their normal appearance and activity during the first day after injection, and by their survival for prolonged periods, the frogs were not damaged by the drug. The assumption is made that intermediary metabolism of organs and tissues other than the motor end plates is not affected by d-tubocurarine.

The closed circuit respirometer is illustrated in figure 1. That part of the apparatus above the dotted lines, as well as that part including stopcock C, manometer, the two tuberculin syringes and part of the tubing going to the thermobarometer were not submerged in the constant temperature water bath. Pumice stones ware used in the carbon dioxide trap to cause small bubble formation of the gas as it was pumped through 100 ml of 20% KOH solution.

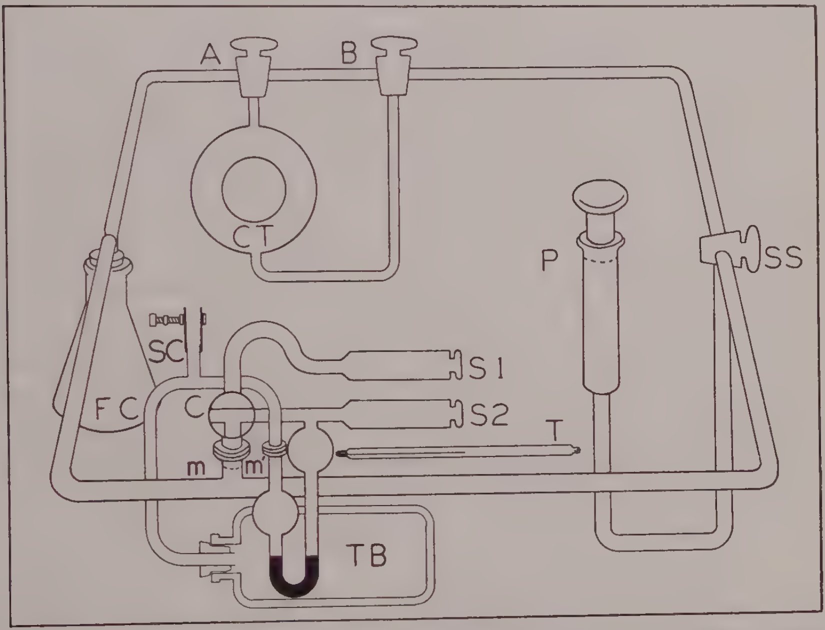

Fig. 1 Diagram of closed circuit respirometer. The electric stirrer, water bath and thermometer for the bath are not shown. FC, frog chamber; CT, carbon dioxide trap, 250 ml Florence flask; P, 50 ml syringe; SC, metal stopcock; S#1, S#2, 1 ml tuberculin syringes; m and m′, metal connections; SS, special two-way stopcock; T, thermometer; A, B, C, three-way stopcocks; dotted line, water level of water bath; TB, thermobarometer attachment. The manometer was filled with kerosene colored with Sudan III.

During a normal day's run one frog from each of the 4 experimental groups was studied. After immobilization with curare the frog was placed in the chamber FC and 30 minutes were allowed for temperature equilibration. The measurements required for calculation of R.Q., oxygen and carbon dioxide were completed in about one hour in three steps. Rate of change in volume with CT out of the circuit gave R.Q. After absorption of carbon dioxide in CT change of volume gave oxygen consumption. Carbon dioxide production was calculated from oxygen consumption and R.Q. All gas volumes were reduced to STP, dry, and results were expressed as milliliters of oxygen or carbon dioxide/100 gm/hr., wet weight of animal.

The whole apparatus and its component parts were checked for accuracy. Volume changes were accurate to ± 1% ml as judged by injection of known volumes of air. Complete absorption of 1.0 ml of carbon dioxide was demonstrated after injection of pure carbon dioxide. All syringes were calibrated by weight.

In arriving at the statistical significance of all results Fisher's "t" test was employed and 5% was considered the fiducial limit of significance.

As a check on the validity of using wet weight the total water content of animals from the 4 groups was measured. Animals kept at room temperature averaged 84.7% total body water; refrigerated animals averaged 84.0%. The difference was not significant.

## RESULTS

*Seasonal variations in the metabolism of frogs.* The data for each group of animals are presented in figures 2, 3 and 4. In addition, certain data were selected which were collected from animals subjected to conditions during a particular season which were comparable to those found in their normal habitat (table 2). Data from Group I (frogs kept at 22°–28°C.; unfed) during March, April and May represent spring frogs; values from Group II (frogs kept at 22°–28°C.; fed) during June, July and August represent summer frogs; values from Group III (frogs kept at 4°C.; unfed) during September, October and November represent autumn frogs; values from Group III (see above) during December, January and February represent winter frogs. Unavoidably, the populations changed in the sense that replacements with new frogs were made when the original frogs perished. Thus, in some cases frogs collected in spring replaced frogs collected in winter, and so on through the seasons. The assumption is made that these replacements would contribute to deviations from the mean, but would react enough like the original animals to justify statistically the grouping we have used.

It is important to emphasize first, that all data presented in figures 2, 3 and 4 and table 2 were collected from both male and female frogs that were completely immobilized with d-tubocurarine chloride (Abbott). A statistical analysis revealed no significant differences between data from male and female frogs. Secondly, although the animals were kept at two different temperatures prior to the experimental runs, the actual respiratory measurements were carried out at 22°–25°C. The temperature did not vary over 0.5°C. during the course of any experimental run.

TABLE 2

*Mean values of monthly data combined into seasons*

| SEASON | $O_2$ CONS.[1] | $CO_2$ PROD. | R.Q. |
|---|---|---|---|
| Spring (Group I) | 3.96 | 3.25 | 0.82 |
| Summer (Group II) | 3.38 | 2.71 | 0.80 |
| Autumn (Group III) | 2.18 | 2.57 | 1.20 |
| Winter (Group III) | 2.39 | 2.77 | 1.16 |

[1] ml/100 gm wet wt./hour, STP.

The oxygen consumption of frogs kept at 22°–28°C. (fig. 2) was maximal in the spring in both the fed and unfed groups and tended to be least in the summer. The animals stored at 4°C. whether fed or unfed had the greatest oxygen consumption in the spring and the least in the fall or early winter. The 4°C. groups were significantly lower than the 22°–28°C. groups at all seasons. Feeding had no apparent effect on oxygen consumption. Values from table 2 show that if we consider the oxygen consumption of spring frogs as being 100%, values for the other seasons are as follows: summer 83%, autumn 78%, winter 60%. Statistically, summer data

(Aug.) differed from autumn (Oct.) values and winter data were significantly lower than spring values. No difference occurred between autumn and winter frogs.

With respect to carbon dioxide production all 4 groups showed similar trends and comparable absolute values from season to season (fig. 3). There were maxima in the spring and minima in the summer or early fall. Feeding had no significant effect and storage at 4°C. but little. The seasonal data (table 2) show only slight differences in carbon dioxide production during summer, fall and winter. Maximal production occurred during the spring.

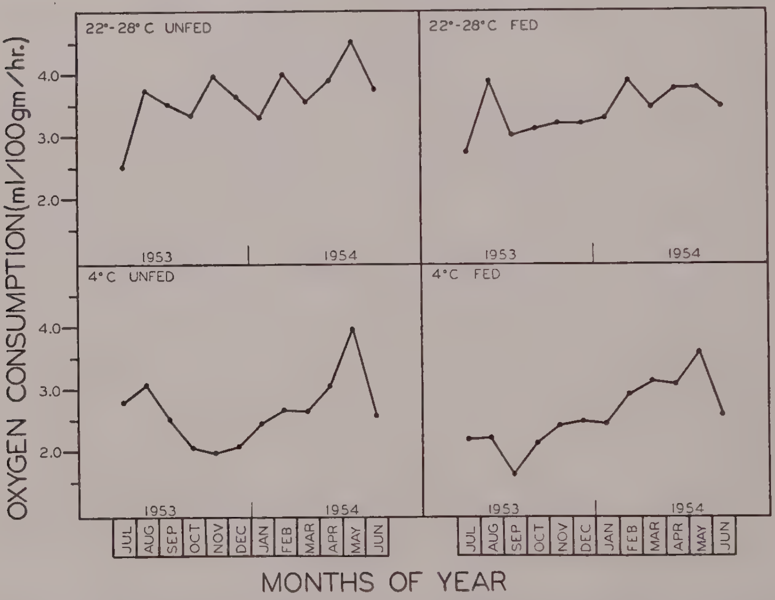

Fig. 2 Oxygen consumption of different groups of frogs, measured at 22°–25°C. All points are averages of groups for the month. All data expressed in ml/100 gm of frog (wet weight)/hour.

Ordinates: ml/100 gm/hour
Abscissae: months
Upper left: Frogs kept at room temp.; no food, Group I.
Upper right: Frogs kept at room temp.; fed, Group II.
Lower left: Frogs kept at 4°C.; no food, Group III.
Lower right: Frogs kept at 4°C.; fed, Group IV.

The R.Q. showed highly significant variations in the 4°C. animals and similar but less striking trends in the 22°–28°C. animals (fig. 4). There were minima in the spring and early fall and maxima during winter or late fall. Feeding had no significant effect; however, refrigeration caused higher values in almost every month. The seasonal data (table 2) show that the R.Q. is maximal during the fall and winter and minimal during spring and summer. Spring frogs showed a significant decrease in R.Q., when compared to winter frogs but there was no difference between spring and summer R.Q. All these differences in R.Q. are the arithmetical result of the fact

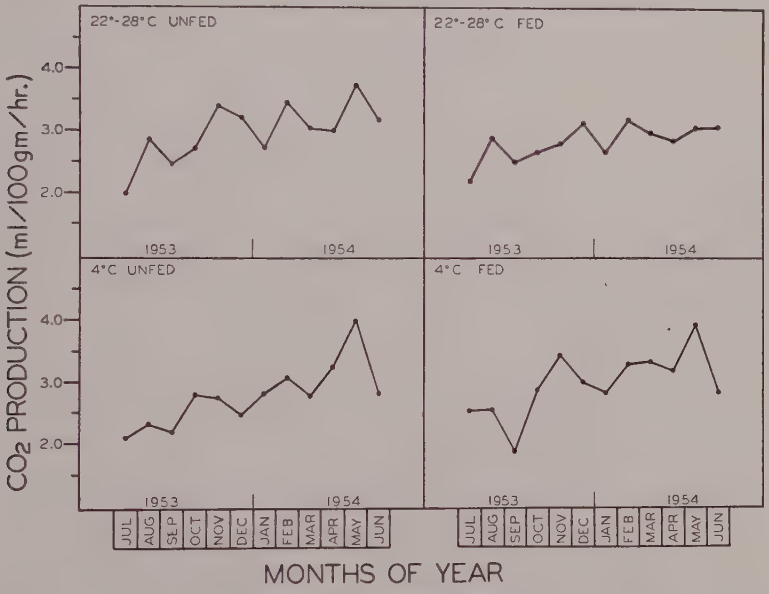

**Fig. 3** Carbon dioxide production of different groups of frogs, measured at 22°–25°C. All points are averages of groups for the month. All data expressed in ml/100 gm of frog (wet weight)/hour.

Ordinates: ml/100 gm/hour

Abscissae: months

Upper left: Frogs kept at room temp.; no food, Group I.

Upper right: Frogs kept at room temp.; fed, Group II.

Lower left: Frogs kept at 4°C.; no food, Group III.

Lower right: Frogs kept at 4°C.; fed, Group IV.

that refrigeration diminished the oxygen consumption of the 4°C. animals in comparison with the 22°–28°C. animals at times when the carbon dioxide production was not different among the groups.

*Effect of temperature on metabolism.* In groups of frogs treated similarly but kept at two different temperatures the

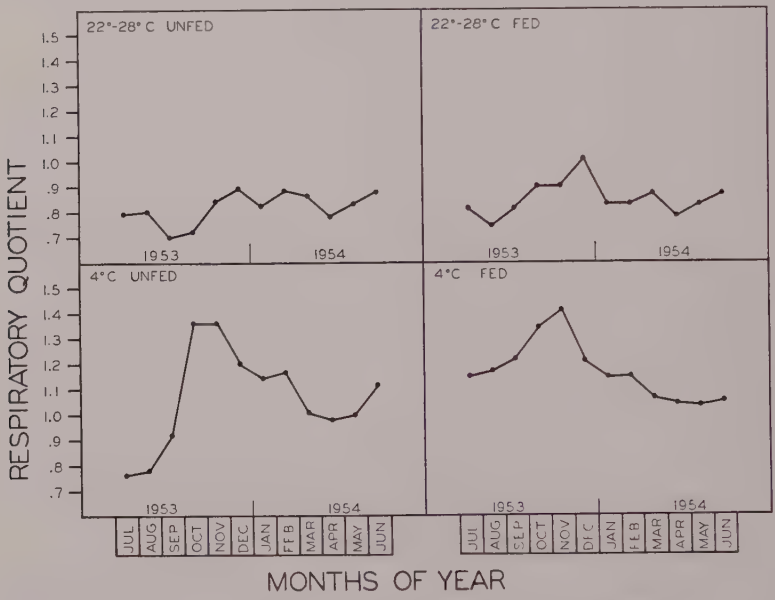

Fig. 4 Respiratory quotient of different groups of frogs, measured at 22°–25°C. All points are averages of groups for the month.

Ordinates: Respiratory Quotient
Abscissae: months
Upper left: Frogs kept at room temp.; no food, Group I.
Upper right: Frogs kept at room temp.; fed, Group II.
Lower left: Frogs kept at 4°C.; no food, Group III.
Lower right: Frogs kept at 4°C.; fed, Group IV.

oxygen consumption of refrigerated frogs was decreased in 23 of 24 comparisons. Unfed frogs kept at 4°C. (July data excepted) showed decreases ranging from 12% to 50% with an average decrease of about 26%. These differences were significant during all months except July, August, May and June. Data for fed frogs showed that refrigeration caused an average decrease in oxygen consumption of about 24% with differences ranging from 7% to 46%. All differences were significant with the exception of data for May, June and July. The carbon dioxide production was much less affected by refrigeration, being significantly decreased in only three of 24 comparisons. As a result of this interesting differential effect of refrigeration upon oxygen consumption and carbon dioxide production, the R.Q. increased during refrigeration, very strikingly in fall and early winter (fig. 4). The values for unfed frogs kept at room temperature were significantly lower than those for unfed, refrigerated animals during all months of the year with exception of July and August. Refrigeration of fed frogs caused a significant increase over values for fed animals kept at room temperature during all months of the year.

*Effect of feeding on metabolism.* A statistical analysis of data from fed and unfed animals kept at the same temperature revealed that in only 4 of 24 comparisons were there significant differences between the oxygen consumption of these groups. In only 4 cases was the carbon dioxide production significantly different in 24 observations and the R.Q. data were only slightly more irregular showing differences in 6 of 24 comparisons.

## DISCUSSION

The values for total oxygen consumption of frogs as determined in this experiment are generally lower than values reported by other workers. This is not surprising since all of the animals in this experiment were completely immobilized during the course of the experimental period, a procedure not followed by previous investigators. Dolk and Postma ('27) and Bohr ('00) were the only workers who collected metabolic data for frogs at a constant temperature during several months of the year and for this reason their data are most comparable to the results presented here. Their data and ours generally agree that oxygen consumption of frogs reaches a maximum in the spring, remains quite high until early fall and is minimal in late fall and early winter. Athanasiu ('00) indicated that maximal values were attained during June and minimal oxygen consumption during November. He conducted experiments on groups of animals rather than on individual frogs during 9 months, and no two groups of data were collected at the same temperature. In view of this fact it is improbable that any valid conclusions concerning seasonal variations in the oxygen consumption of frogs can be drawn from his data.

Holzapfel ('37) placed frogs *(Rana pipiens)* in a refrigerator at 0°C. each month of the year and found that only those frogs which were placed in the refrigerator from October to April became completely torpid and buried themselves in the sand in characteristic hibernating position. It seems valid, therefore, to assume that fluctuations in the oxygen consumption of frogs as shown in the present work are true seasonal changes and not simply changes brought about by subjecting the frogs to different temperatures.

Most of the previous studies on carbon dioxide production for frogs show that variations occur which parallel fluctuations in the oxygen consumption. In the present work only slight variations occurred during summer, fall and winter, but maximum production occurred in the spring.

In the past, R.Q. data for frogs have been quite contradictory; no set values for any given season have been agreed upon. The present experiment may suggest possible explanations for some of these irregularities, in that refrigeration uniformly caused an increase in the R.Q. Therefore, the conditions under which frogs are kept prior to an experimental period may have a marked effect on the R.Q. Kayser ('40) has presented evidence that changes in temperature and carbon dioxide tension during the course of an experiment will cause alterations in the observed R.Q.

Perhaps the most surprising result presented here is the absence of increased oxygen consumption as a result of feeding. Bohr ('00) noted that there was no correlation between oxygen consumption and food intake in frogs. He found that spring frogs with no food in their stomachs or intestines had as high or higher rates of metabolism as summer frogs whose alimentary tracts contained large amounts of partially digested food materials. In contrast to these results, Elsas ('13) found with frogs notable increases in metabolism after protein and carbohydrate feeding, but small increases following fat feeding. Benedict ('32) also noted large increases in oxygen consumption and heat production of large cold-blooded animals after feeding. Warren ('40) found that *Rana pipiens* fed beef liver pulp consumed about 6.36 ml oxygen/100 gm/hr., whereas starved controls consumed about 4.40 ml/100 gm/hr. at the same temperature. We have no explanation for the discrepancy between our results and those of others, unless it may be that the fed frog is more active than the unfed. Our curarized frogs could not move while measurements were in progress; those of others presumably could move about.

In explaining the decrease in oxygen consumption in animals stored at 4°C. as compared with animals stored at 22°–28°C. even when oxygen consumption was measured at 22°–25°C. in all cases, the previous literature is of little help. Our results confirm those of Long ('52) for *Rana pipiens*. Hill ('11) using direct calorimetry measured the heat production of frogs and other cold-blooded animals at various temperatures. He postulated a high temperature coefficient as an explanation of how it is possible for poikilotherms to live for long periods in the cold without food. Scholander et al. ('53) have reviewed the literature with respect to seasonal adaptations in the metabolism of poikilotherms and state that in no case has it been shown that organisms are adapted to seasonal or other fluctuations in temperature by having a low respiratory $Q_{10}$, i.e., by being metabolically insensitive to temperature changes. The opposite view is defended by Rao and Bullock ('54), who believe that $Q_{10}$ of a given species may vary with the habitat temperature when size as a variable is ruled out.

It is likely that the frog may be different from many other cold-blooded forms in their adaptation to cold. For instance, Scholander et al. ('53) demonstrated that in a number of closely related lamellibranchs taken from arctic and warmer waters, the arctic forms as a rule have higher oxygen consumption than the southern forms when measured at the same intermediate temperature. In contrast, our frogs' oxygen consumption decreased when they were refrigerated. It is tempting to postulate enzymatic, cellular or endocrinological changes to explain adaptive changes, but clear evidence is scanty. Studies such as those of Precht ('51) on adaptive changes in enzymes in relation to habitat temperature may eventually provide the explanation for our findings and those of others.

## SUMMARY

1. Oxygen consumption, carbon dioxide production and R.Q. of *Rana pipiens* were measured monthly throughout one year in a closed circuit respirometer maintained at a temperature of 22°–25°C. Two groups of frogs were starved and two groups were force fed every 5 days with homogenized tuna fish. One fed and one unfed group were kept at 22°–28°C.; the other two groups were kept at 4°C. except during measurement of respiration. To eliminate possible influences of muscular activity all animals were curarized prior to measurement.

2. A seasonal variation was observed in oxygen consumption, which reached a maximum during the spring and decreased some 15% during the summer. During fall, the oxygen consumption was only slightly decreased from summer values but winter frogs showed a decrease of approximately 40% from spring values.

3. The carbon dioxide production was maximal in the spring.

4. As the mathematical result of differential changes in oxygen consumption and carbon dioxide production, the R.Q.

varied widely with season. Autumn frogs had a very high R.Q. (mean of 1.20) which was higher than values for the other seasons. During the winter the R.Q. remained high but showed a gradual decline so that by spring it averaged approximately 0.82. No significant difference occurred between the R.Q. of spring and summer frogs.

5. Refrigeration of unfed frogs at 4°C. caused the oxygen consumption at 22°–25°C. to be decreased an average of 26% during each month of the year with exception of July. Refrigeration of fed frogs caused an average decrease in oxygen consumption of about 24% when compared to fed animals kept at room temperature.

6. Refrigeration had little effect on the carbon dioxide production of these *Rana pipiens*. As a result, changes in R.Q. were dependent primarily upon changes in the oxygen consumption and not on changes in the carbon dioxide production. The R.Q. was substantially increased by refrigeration, especially in fall and early winter.

7. Feeding had practically no effect on the oxygen consumption, carbon dioxide production or R.Q. of *Rana pipiens* at any time of year.

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# THE PHOSPHATASE ACTIVITIES OF THE MICROSOMAL (SUBMICROSCOPIC PARTICULATE) FRACTION PREPARED IN AN ELECTROLYTE MEDIUM[1,2]

N. SONNENSCHEIN AND M. J. KOPAC
*Biology Department, New York University*

FOUR FIGURES

Isolation of cellular particulates is generally achieved by the centrifugal fractionation of tissue homogenates. Submicroscopic granules, which include the microsomes, have been isolated by this method, and their biochemical properties have been studied in an attempt to determine the role of these particulates in the living cell. A more complete understanding of this vital problem, however, is hindered by the structural heterogeneity of these granules, and by their biochemical alteration in various homogenizing media.

Relatively little is known regarding the enzyme content of the submicroscopic particulates. Several enzymes, however, such as adenyl esterase (Omachi, Barnum and Glick, '48), adenosine triphosphatase (Steinbach and Moog, '45), and glucose-6-phosphatase (Swanson, '51), have been localized predominantly in these particulates. Alkaline phosphatase activity has been associated with the microsomes of the rat kidney (Kabat, '41), and of the intestinal mucosa of the cow (Harris, Bergren, Bavetta and Mehl, '52). The presence of alkaline phosphatase in all sedimentable particles isolated from mouse liver homogenates was noted by Chantrenne

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('47), but the relative phosphatase activities of the granules differed with the method of expression of enzymatic activity, i.e., nitrogen content, or ribonucleic acid content. More recently, Kallman ('51) noted that the submicroscopic particulates isolated from rabbit intestinal mucosal homogenates were more active than the microscopic in the hydrolysis of a number of phosphorylated esters. The work indicated that alkaline phosphatases may not be one, but rather a group of individual enzymes, each specific towards its particular substrate.

The rate of enzymatic activity is modified by the acidity of the medium, and pH optima have been demonstrated for alkaline phosphatases in various tissues (Reis, '51). The pH optima, however, were found to be different depending on the substrate utilized (Walker and King, '50). The concentration of substrate also affected the pH at which maximal enzymatic activity was observed (Ross, Ely and Archer, '51), i.e., as the molarity of the substrate was decreased, the pH optimum fell.

In view of these considerations, there is a need for continued evaluation of the effects of experimental variables on enzymes, such as phosphatases, whose status is not well-defined. Accordingly, this investigation was undertaken to determine the effects of: (1) substrate, (2) substrate concentration, and (3) pH, on the phosphatase activities within submicroscopic particulates obtained from liver homogenates by differential centrifugation. It was hoped that the results of the study might also aid in the elucidation of the intracellular distribution of phosphatases.

## MATERIALS AND METHODS

Adult frogs of either sex, *Rana pipiens*, stored at 5°C. were used in the investigation. After decapitation, the liver was immediately removed, cut into small strips, and washed free of blood in several changes of Kassel's solution (Kassel and Kopac, '53) containing 0.042 M KCl, 0.017 M NaCl, 0.001 M $CaCl_2$, and 0.001 M $MnCl_2$. The liver was weighed and placed in a glass homogenizer containing 4 ml of Kassel's solution per gram of tissue. To avoid heating effects, homogenization was carried out at 0°C. Aliquots of the crude homogenate, prepared in this manner, were then subjected to centrifugal fractionation at 0°C., in the International Portable Refrigerated Centrifuge, model PR-1, equipped with the high speed head.

*Particulate fractionation.* A modification of the method of Kallman ('51) was used for the centrifugal fractionation of the liver tissue. Initially, the crude homogenate was centrifuged three times at 1,500 g to sediment unbroken cells, nuclei, and debris. The final suspension was termed the refined homogenate, and from it, the microsomal fraction was ultimately segregated.

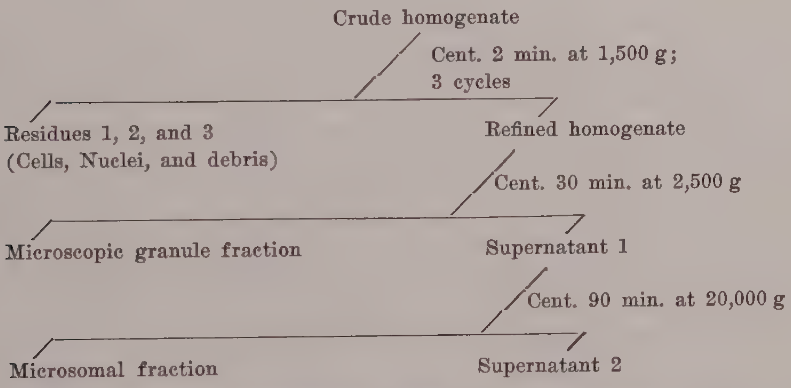

Fig. 1 Centrifugation procedure followed in the preparation of the microsomal fraction.

The refined homogenate was centrifuged at 2,500 g for 30 minutes. This brought down the large granules which were discarded. The supernatant was then spun at 20,000 g for 90 minutes to sediment the microsomal particulates. After discarding the supernatant, the final residue was resuspended in 4 ml of Kassel's solution and stored at 4°C. The complete procedure for the isolation of the microsomal fraction is given in figure 1.

The compounds which served as substrates were glucose-1-, glucose-6-, and fructose-6-phosphate, as well as adenosine-3-, and adenosine-5-phosphate. These substrates were chosen because they are normally present in the cell. Each was prepared at 5 concentrations extending from 0.000127 M to 0.021 M, and buffered by Veronal-HCl over a pH spectrum ranging from 6.8 to 9.6. It was necessary to convert adenosine-3- and adenosine-5-phosphate, as well as glucose-6-phosphate, to the more soluble sodium salt in order to prepare solutions of the required molarities.

*Reaction mixture.* A 50 μl sample of the enzyme preparation, i.e., crude homogenate or microsomal fraction, was transferred by a Levy constriction pipette (Levy, '36) to a reaction vessel containing 50 μl of buffered substrate. The solutions were mixed by an electromagnetic stirrer, and the reaction vessel was then stoppered and immersed for 15 minutes in a water bath at 37°C. Enzymatic activity was terminated by the addition of 20 μl of chilled 10% trichloroacetic acid containing 0.75% uranium nitrate. The protein and nucleotide precipitates were centrifuged at 5,000 g for 5 minutes, and the reaction vessel was then stored at 4°C. until the determination of inorganic phosphate was made.

*Determination of enzymatic activity.* Phosphatase activity was determined by the amount of inorganic phosphate released from a substrate. A micro-method was necessary for the measurement of inorganic phosphate, since the volume of the microsomal fraction never exceeded 5 ml. Comparison of the micro-methods available, revealed that the procedure of Holter and Li ('51) was most suitable for this study. With this technique, measurements of inorganic phosphate ranging from 0.019 to 0.45 μg phosphorus were possible.

Inorganic phosphate was measured colorimetrically with a microphotometer modified after those described by Commoner ('48), Krugelis ('50), and Glick ('51). The photometric apparatus consisted of a lamp (Leitz Monla, 5V, 6A), a condensing system giving a narrow parallel beam, a microscope, a beam splitter from a Leitz Micro-ibso attachment, and a photocell with a suitable amplifier and galvanometer (Photovolt 512-M). The microscope objective (Leitz no. 2) was focussed at the midpoint of a cuvette 7 mm in height and 3 mm in bore. At this focal point, the diameter of the light beam was approximately 1 mm.

An aliquot of each enzyme preparation was analyzed for nitrogen by the micro-Kjeldahl procedure of Ma and Zuazaga ('42). Final activities were expressed as micromole phosphate, per 15 minutes, per microgram nitrogen.

## RESULTS

The effect of pH on the phosphatase activity of the microsomal fraction was essentially the same in the presence of each substrate. Thus, figure 2 will be sufficient to illustrate this relationship. In general, the pH optima for maximal enzymatic activity were found to vary with different substrates. In addition, individual substrates showed a different optima for enzymatic activity depending on concentration. In every instance, distinct pH optima were obtained with high substrate concentrations, but as substrate concentration was lowered, the pH optima were not as sharply defined. Overall hydrolytic activity also declined with decreased substrate molarity.

The potency of a microsomal fraction in liberating phosphate from a substrate varied with each preparation. With every substrate whose concentration equalled or exceeded 0.0058 M, however, the pH at which maximal activity was observed, was essentially similar (table 1). For example, at 0.021 M, the optimal pH with every substrate but glucose-1-phosphate was 9.4 or 9.5. Enzymatic activity with 0.0127 M glucose-1-phosphate, however, reached its peak at a pH of 9.1, while maximal activity with this substrate, at a concentration of 0.006 M was attained at pH 8.5 (table 1).

*Enzymatic activity with individual substrates.* Phosphatase activity of the microsomal fraction was most vigorous in the presence of glucose-1-phosphate, 0.0201 M at pH 9.2. When the substrate concentration was lowered to 0.0127 M, the amount of inorganic phosphate liberated was essentially the same since the measured quantity of phosphate released differed by only 0.018 μM (table 2). This indicated that the enzyme system was approaching its maximal activity. At lower concentrations, the amounts of phosphate released enzymatically were less, and the optimal activities occurred at lower pH levels (fig. 2).

TABLE 1

*The pH optimum for maximal phosphatase activity of the microsomal fraction with various concentrations of substrate*

| SUBSTRATE | 0.021 M | 0.0127 M | 0.006 M | 0.00127 M | 0.000127 M |
|---|---|---|---|---|---|
| Glucose-1-phosphate | 9.2 | 9.1 | 8.5 | 8.0 | 7.5 |
| Glucose-6-phosphate | 9.4 | 9.3 | 8.6 | 8.3 | 7.6 |
| Fructose-6-phosphate | 9.5 | 9.3 | 8.6 | 8.2 | 7.8 |
| Adenosine-3-phosphate | 9.4 | 9.3 | 9.0 | 8.3 | 7.6 |
| Adenosine-5-phosphate | 9.4 | 9.3 | 9.0 | 8.3 | 7.8 |

TABLE 2

*A comparison of the phosphatase activity of the crude homogenate and of the microsomal fraction, with various substrates. Enzymatic activity is expressed as micromole phosphate, per 15 minutes incubation at 37°C., per microgram nitrogen.*

| SUBSTRATE | 0.021 M | 0.0127 M | 0.006 M | 0.00127 M | 0.000127 M |
|---|---|---|---|---|---|
| Glucose-1-phosphate | | | | | |
| Microsomal fraction | 0.220 | 0.202 | 0.184 | 0.051 | 0.016 |
| Crude homogenate | 0.178 | 0.164 | 0.074 | 0.019 | 0 |
| Glucose-6-phosphate | | | | | |
| Microsomal fraction | 0.121 | 0.108 | 0.079 | 0.021 | 0.011 |
| Crude homogenate | 0.032 | 0.028 | 0.009 | 0 | 0 |
| Fructose-6-phosphate | | | | | |
| Microsomal fraction | 0.194 | 0.173 | 0.081 | 0.037 | 0.020 |
| Crude homogenate | 0.102 | 0.081 | 0.049 | 0 | 0 |
| Adenosine-3-phosphate | | | | | |
| Microsomal fraction | 0.179 | 0.168 | 0.077 | 0.054 | 0.008 |
| Crude homogenate | 0.089 | 0.057 | 0.038 | 0 | 0 |
| Adenosine-5-phosphate | | | | | |
| Microsomal fraction | 0.178 | 0.169 | 0.038 | 0.051 | 0.007 |
| Crude homogenate | 0.092 | 0.050 | 0.040 | 0 | 0 |

Fructose-6-phosphate was readily hydrolyzed by the microsomal fraction. The enzymatic activity with this substrate was quite high, although it was exceeded by the enzyme preparation in the presence of glucose-1-phosphate (table 2). At the lowest concentration (0.000127 M), however, phosphatase

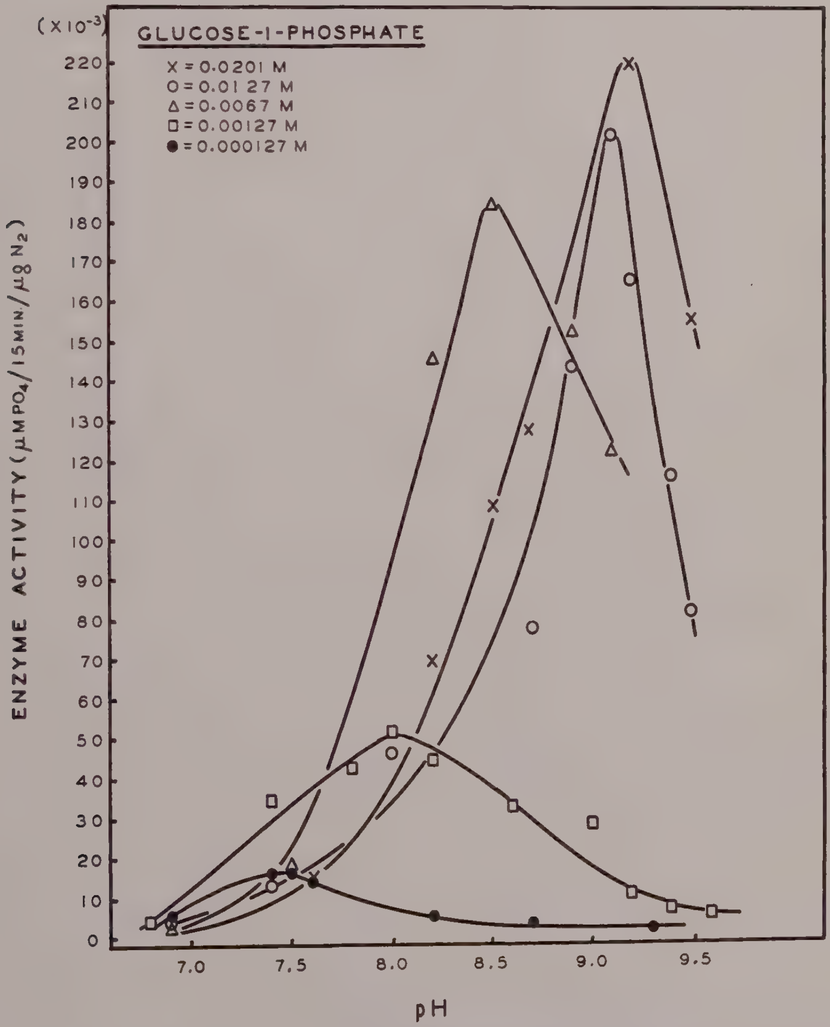

Fig. 2 The effect of pH on the phosphatase activity of the microsomal fraction with various concentrations of substrate.

activity of the microsomal fraction was more pronounced with fructose-6-phosphate than with any other substrate of equivalent molarity (table 2). The most alkaline medium required for optimal enzymatic activity, namely 9.5, was also noted with fructose-6-phosphate at a concentration of 0.0201 M.

In the presence of glucose-6-phosphate, the phosphatase activity of the microsomal fraction was lowest at every concentration (table 2).

When either adenosine-3- or adenosine-5-phosphate served as the substrate, the quantity of inorganic phosphate enzymatically liberated was approximately equal at all concentrations. Considerable enzymatic activity was shown by the microsomal fraction in the presence of large amounts (0.006 M or higher) of these nucleotides (table 2). At a concentration of 0.000127 M, however, enzymatic hydrolysis of either compound was only slight (table 2).

*Correlation of pH, substrate concentration, and enzymatic activity.* Rather alkaline media (pH 9.2–9.5) were necessary for maximal phosphatase activity of the microsomal fraction with high substrate concentrations, but as substrate molarity decreased, optimal enzymatic activity was noted at less alkaline levels. The pH optima of enzymatic activity of the microsomal fraction were essentially the same, however, with all substrates of comparable concentration (table 1).

The effect of substrate concentration on enzymatic activity was essentially the same with all substrates. Therefore, a single curve, figure 3, will suffice to exemplify this relationship. Enzymatic activity was proportional to and dependent on substrate concentration, at the lower concentrations, i.e., 0.000127 and 0.00127 M. With concentrations of 0.0127 M or higher, however, the reaction rate became independent of substrate molarity, and the curve levelled off (fig. 3). At this point, interaction between enzyme and substrate molecules is approaching its maximum, and further increase in substrate concentration will have little effect.

*Phosphatase activity in the crude homogenate.* In order to determine if the enzyme system was predominantly localized in the microsomal fraction, the phosphatase activity of the crude homogenate was also measured. Enzymatic activity, with varying substrate concentration and pH, differed only in quantitative manner from that exhibited by the microsomal fraction.

Phosphatase activity of the crude homogenate was most vigorous with glucose-1-phosphate, while enzymatic activity was lower in the presence of fructose-6-, adenosine-3-, and adenosine-5-phosphate. Substrate hydrolysis by the phosphatases of the crude homogenate was lowest with glucose-6-phosphate (table 2).

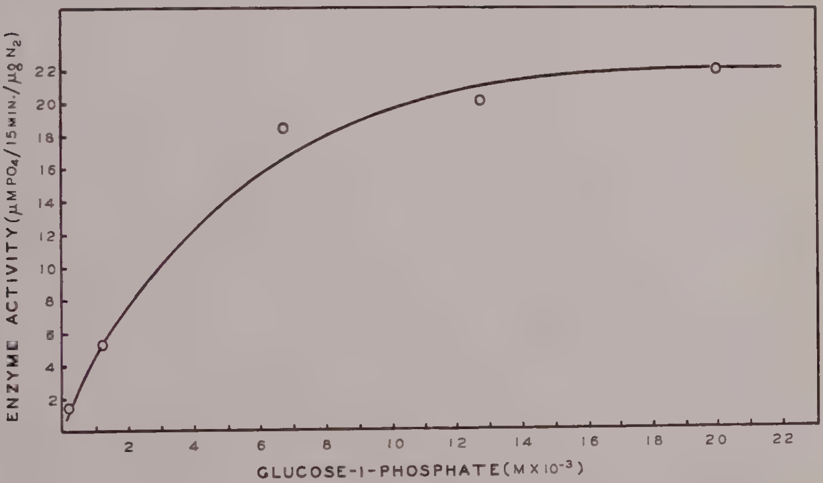

Fig. 3 The effect of substrate concentration on the rate of phosphatase activity of the microsomal fraction.

At comparable substrate concentrations, the phosphatase activity of the crude homogenate was consistently lower than that noted for the microsomal fraction (table 2, fig. 4). For example, at a concentration of 0.00127 M, hydrolysis of only one substrate, *viz.*, glucose-1-phosphate, could be detected. With all other substrates, however, higher concentrations (0.006 M) had to be used before phosphatase activity could be demonstrated in the crude homogenate (table 2). In addition, the pH optima for maximal phosphatase activity of the crude homogenate with substrate concentrations of 0.006 and 0.0127 M, were not as well-defined as those pH optima noted with the microsomal fraction at these concentrations (fig. 4).

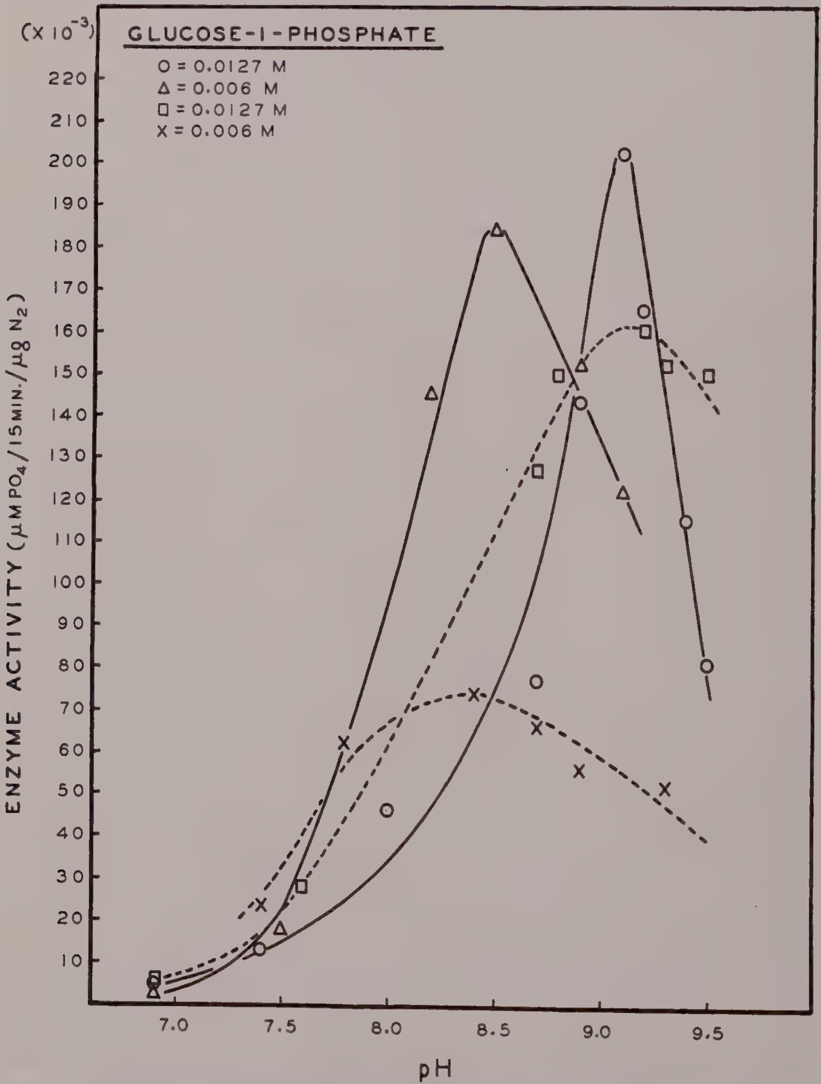

Fig. 4 Phosphatase activity of the microsomal fraction and crude homogenate, at various pH levels, with 0.006 M and 0.0127 M glucose-1-phosphate. The curves for the microsomal fraction are represented by solid lines (———), while the broken lines (– – – –) represent the crude homogenate.

## DISCUSSION

In the present study, the most vigorous response of the enzyme preparations was elicited with glucose-1-phosphate, while activity was lowest in the presence of glucose-6-phosphate. Similar results were obtained by Kallman ('51) on submicroscopic particulates isolated from homogenates of rabbit intestinal mucosa. Hers, Berthet, Berthet and de Duve ('51), however, observed that the enzymatic activity of the microsomes was highest with glucose-6-phosphate. These investigators prepared the particulate fraction in hypertonic sucrose, while in our work, the homogenizing medium was an electrolyte solution. Enzymatic distribution among isolated particulates may differ, therefore, depending on the choice of homogenizing media.

The transfer of phosphate from the first to the 6th carbon of glucose is catalyzed by phosphoglucomutase. There was, however, no evidence of such a transfer in this investigation. If such a change had occurred, the phosphatase activity of the enzyme preparations should be equal with either substrate, since the addition of glucose-1-phosphate would be, in essence, the addition of glucose-6-phosphate. The results do indicate, however, that a glucose-1-phosphatase may be present in the microsomal fraction of frog liver.

Enzymatic hydrolysis of fructose-6-phosphate by the microsomal fraction is almost equal to the hydrolysis of glucose-1-phosphate. Possibly, a fructose-6-phosphatase may be present in frog liver. It is also conceivable that the phosphate group of fructose-6-phosphate could be transferred to the number one carbon of alpha D-glucose by means of a phosphotransferase. Glucose-1-phosphate so formed, could then be acted on by glucose-1-phosphatase. Meyerhof and Green ('50) noted that alkaline phosphatase catalyzed the transfer of phosphate from glucose-1-phosphate to fructose. The reverse may be equally true. Such an enzyme may act in conjunction with alkaline phosphatase, or as Meyerhof believed, be identical with it.

In an investigation of the enzymatic properties of particulates isolated from rabbit liver, Hers, Berthet, Berthet and de

Duve ('51) found that glucose-6-phosphatase was predominantly localized in the microsomal fraction. The submicroscopic particulates isolated from frog liver, however, did not show this high glucose-6-phosphatase activity in this study. It may be that glucose-6-phosphatase, in frog liver, is associated with some particulate fraction other than the microsomes. Swanson ('51), however, noted that maximal activity of glucose-6-phosphatase occurs at pH 6.5 in the soluble fraction (which includes the microsomes) of rat liver. Since this pH is below the lowest value in this investigation, it is possible that analysis of the microsomal fraction for enzymatic activity at a pH of 6.5 might result in its detection. Another possibility for the low glucose-6-phosphatase activity observed in our enzyme preparations may be the adsorption of the enzyme system by other particulates during centrifugal separation.

A specific 5-nucleotidase that acts only on nucleotides in which the phosphate group is attached to the number 5 carbon of the pentose, has been noted by several investigators (Heppel and Hilmoe, '51). In the present investigation, however, the reaction rates of the enzyme preparations were equal, with every concentration of either adenosine-3- or adenosine-5-phosphate; the presence, therefore, of an enzyme specific for either substrate could not be established. Possibly, such an enzyme may be associated with other cytoplasmic granules.

The phosphatase activity of the crude homogenate obtained from frog liver was lower than might be expected, on the basis of the phosphatase activity exhibited by the microsomal fraction. In this investigation, as well as others, enzymatic activity was expressed in terms of nitrogen content. Using paper electrophoresis, Journey ('54) demonstrated that much of the protein of the crude homogenate isolated from frog liver was not enzymatic in function. Thus, the phosphatase activity of the crude homogenate would be higher if the nitrogen content was disregarded. Enzymatic activity, expressed in terms of nitrogen content may, therefore, lead to erroneous conclusions, unless more purified enzyme preparations are utilized. The interpretations of results obtained from studies on the enzymatic distribution of cellular particulates may also differ, depending on how enzymatic activity is expressed.

Innumerable investigations of alkaline phosphatase activity, *in vitro,* have been made. Most of the studies, however, were carried out using relatively high substrate concentrations. It is highly improbable that the living cell would contain large amounts of substrate of the order of 0.01 M, for example. Also, in most studies, the pH levels of the media were in the alkaline range in order to achieve maximal enzymatic activity.

In this investigation, when substrate concentrations were decreased, the enzyme preparations showed optimal activity as the pH levels approached neutrality. This was also demonstrated by Ross, Ely and Archer ('51) with rat liver extracts. These results suggest that the pH optimum of intracellular phosphatases may be near that of the living cell, at physiologically significant substrate concentrations.

The effect of experimental conditions on enzymes whose status may not be well-defined should be thoroughly investigated. This is especially true for enzymes such as phosphatases, whose specificity as yet, has not been confirmed. It is apparent, therefore, that any future study of phosphatases, should be carried out not at a single pH, but rather over a broad spectrum. In addition, the reaction rates of this enzyme system should be studied over a wide range of substrate concentration; and if we are to attempt to relate phosphatase activity in particulate fractions to its activity in the living cell, it would be advisable also to select substrates known to be present in the cell.

## SUMMARY

1. An investigation was made of the phosphatase activities of the microsomal fraction isolated from frog liver homogenates by centrifugal fractionation. The enzyme preparations were made up in an electrolyte medium containing 0.042 M KCl, 0.017 M NaCl, 0.001 M $CaCl_2$, and 0.001 M $MnCl_2$.

2. Each substrate, i.e., glucose-1-, glucose-6-, and fructose-6-phosphate, as well as adenosine-3-, and adenosine-5-phosphate, was prepared at 5 concentrations over a pH range from 6.8 to 9.6.

3. Phosphatase activity was highest with glucose-1-phosphate and lowest with glucose-6-phosphate, indicating the microsomal fraction of frog liver may contain a glucose-1-phosphatase which differs from glucose-6-phosphatase. Enzymatic activity was essentially the same, however, with either adenosine-3- or adenosine-5-phosphate, and the presence of either a specific 3- or 5-nucleotidase, cannot be established.

4. Distinct pH optima, in the alkaline range, were obtained with high substrate concentrations. With decreased substrate concentrations, however, the pH optima were not as sharply defined, and optimal enzymatic activity was demonstrated at less alkaline pH levels. Thus, the pH optimum of intracellular phosphatases may be near that of the living cell, at physiologically significant substrate concentrations.

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# SOME RELATIONS BETWEEN RESISTIVITY AND ELECTRICAL ACTIVITY IN THE CEREBRAL CORTEX OF THE CAT[1]

W. H. FREYGANG, JR. AND W. M. LANDAU
*Laboratory of Neurophysiology, National Institute of Mental Health,*
*National Institutes of Health, Public Health Service,*
*U. S. Department of Health, Education, and*
*Welfare, Bethesda, Maryland*

SEVEN FIGURES

## INTRODUCTION

At this time, no report of the measurement of membrane potentials in cortical neurones with micropipettes relates the magnitude of the potential difference across the membrane with the anatomical location of the electrode in the neurone. On the basis of information obtained with gross electrodes, Libet and Gerard ('41) and Bishop ('49) suggested that the membrane potential of neurones in the cortex varies with distance from the pial surface.

In the polarized squid giant axon, Cole and Baker ('41) and Cole and Curtis ('41) showed that a decrease in membrane resistance occurs at a cathode, and an increase at an anode. It has been assumed that this property is common to all neural structures. Gradients of membrane potential along the extent of cortical neurones should produce an extracellular current flow that would similarly influence membrane resistance. Such relations have been sought in this study by resistivity measurements of cortex and white matter.

Four methods were used to alter the potential difference across the cortex: spreading depression, polarizing currents, direct stimulation of the cortex, and indirectly induced cortical activity. Of these, spreading depression produced the largest resistivity change and was studied most extensively.

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[1] A preliminary report of a part of this study appeared in Fed. Proc., *13:* 48, 1954.

While this study was in progress, Leão and Martins Ferreira ('53) reported an increase in the cortical impedance of the rabbit during spreading depression. They used a Wheatstone bridge with sinusoidal currents of variable frequency and measured between the surface of the cortex and a remote point. A similar result with the Wheatstone bridge has been obtained in an isolated slab of a cat's cortex by Grafstein ('54b).

## METHODS

In preliminary experiments, impedance across the cortex was measured between a surface pore electrode and a subcortical electrode, both electrodes having $A_g$–$A_g$Cl junctions. A Wheatstone bridge with a sinusoidal current of 1 Kc. was used.

The Wheatstone bridge method has several disadvantages. Large electrodes are necessary to obtain low enough electrode resistance for sufficient sensitivity, and anatomical localization of the resistance change is accordingly vague. There is a question of whether or not current densities can be maintained low enough to avoid excitation and gross alteration of surrounding cells. Finally, it is awkward to use the same electrodes for recording electrical activity, and simultaneous measurement is difficult.

In order to reduce these difficulties, another method was evolved for measuring resistivity (fig. 1). In principle, the method consists of applying a constant current pulse across the cortex; the potential difference recorded is taken as a measure of resistivity. A square wave of voltage that varied in duration from 0.3 to 0.7 msec. was led to a phase splitter of high output impedance (greater than 20 K). Pulse duration was determined by the time required for the recorded voltage pulse to reach its peak. The upper pulsing electrode was a platinum-platinum black coil placed about 2 cm above the cortical surface in a flowing pool of modified Tyrode's solution at 37°C. The deep electrode was in the pharynx. This arrangement was adopted in order to approach a constant current density with parallel lines of flux normal to the pial surface. The resistance between these electrodes was about 500 ohms and the current pulse was about 3.5 mA or less. A modification of the method of Marshall et al. ('51) was used in the cortical exposure and pool construction.

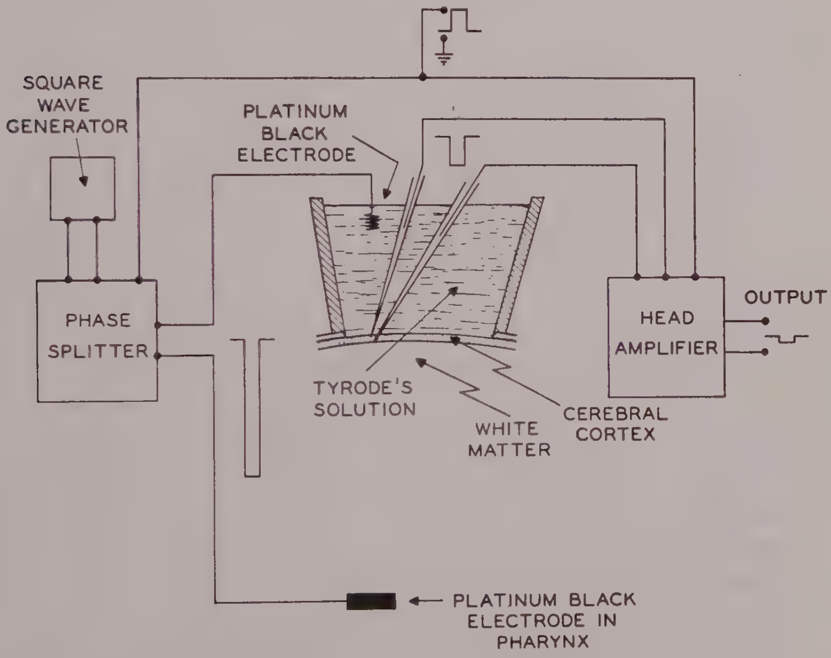

Fig. 1 Schematic diagram of the method for measuring resistivity.

The cortical electrical activity and the voltage pulses produced by the pulsing electrodes were recorded from two glass capillary pipettes of 20–25 μ tip diameter filled with Tyrode's solution. A 15 μ steel wire was inserted into the constricted portion reducing the electrode resistance to 0.1–0.5 megohms. Another 15 μ steel wire glued externally onto the shaft of the electrode was used for electrolytically depositing iron to mark the electrode position (Marshall, '40).

The amplitude of the voltage pulse between the pipette tips is proportional to the resistivity of that portion of the cortex lying between, since the current density is practically independent of the cortical resistance. The voltage drop produced across the entire cortex by the constant current pulse was 20 mV or less. This evoked no evidence of excitation except in some experiments with surface positive polarization.

The major portion of the voltage pulse recorded between the pipettes was opposed by a calibrated pulse led from the phase splitter into the head amplifier. By observing only the difference between these pulses on the cathode ray tube, the sensitivity of the method was increased.

A cathode follower using 954 acorn pentodes was used as the input stage (Dickinson, '50). The direct coupled amplifier had a 2,000:1 rejection ratio of common mode to differential mode signal.

The spreading depression experiments were done under Tyrode's solution in which the potassium concentration had been raised to 27.0 meq./1 replacing sodium. The higher potassium ion concentration made the cortex more susceptible to spreading depression (Marshall, '53). Most other experiments were done under Tyrode's solution from which the potassium had been omitted to reduce susceptibility to spreading depression.

A check upon the ideal of parallel lines of current flow was made by manipulating the pipettes in the pool and in the brain (fig. 2). No pulse was recorded when the pipette tips were at equal depths. Vertical displacement of either one in a uniform medium (Tyrode's solution or white matter) produced a proportional change in the recorded pulse amplitude. This proportionality was limited by the inaccuracy of electrode placement, i.e., 5–10% for movements of 1 mm. The resistivity of the tissue lying between the pipette tips could be calculated by comparing the amplitude of the recorded voltage pulse with the amplitude of the voltage pulse across a measured portion of the Tyrode's pool (resistivity 52 ohm cm at 37°C.).

Transcortical polarization was produced by a battery in series with a selenium rectifier. The rectifier was necessary to prevent shunting of the constant current pulse through the polarizing circuit. A current of about 3 mA was passed between two $A_g$–$A_g$Cl electrodes, one within a millimeter above the surface and the other about 1 cm below the surface of the lateral gyrus.

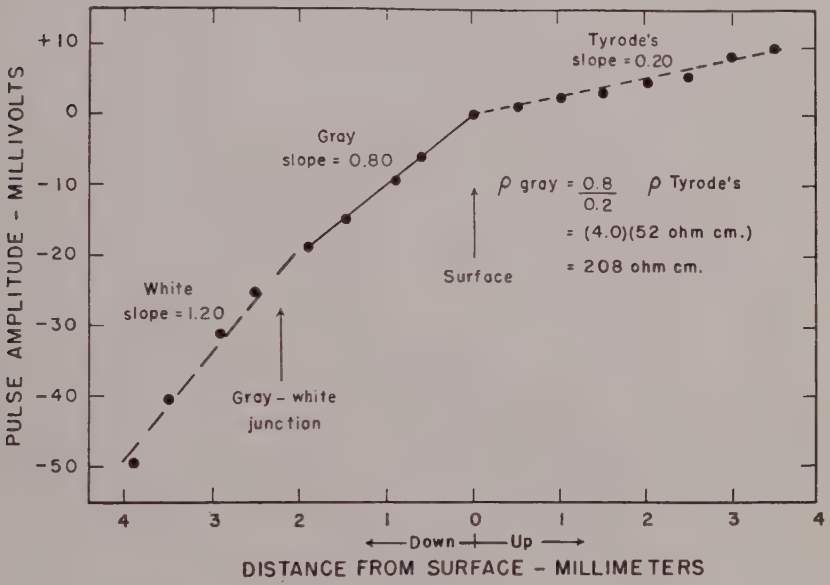

Fig. 2 Measurement of tissue resistivity by the constant current pulse method. With one pipette at the cortical surface, the other was raised through white matter, cortex, and the Tyrode's pool. The voltage pulse appearing between the electrodes is plotted against the distance separating them. Electrode positions in the brain were marked by iron deposition. The resistivity of gray or white matter is estimated as shown.

All observation were made in the lateral and suprasylvian gyri of cats maintained under pentobarbital. Variations produced by respiratory motion of the brain were minimized when necessary by curarization, pneumothorax, and artificial respiration.

## RESULTS

### *Cortical resistivity*

The mean of 17 resistivity measurements made exclusively across whole cortex was 222 ± 9 (S.E.) ohm cm. Comparison of the pulse amplitudes across more superficial cortical layers with those across deeper layers showed no consistent difference in resistivity by this gross approach (fig. 2). Since the electrolytically marked electrode positions are accurate only to 50–100 μ, it was not possible to determine the resistivity of individual cortical layers. Resistivity of white matter was found to be approximately 1.5 times greater than that of the gray. All results were independent of the polarity of the constant current pulse.

Changes in resistivity are presented as the per cent change from the resting (or unstimulated) state.

In fresh preparations, the cortical resistivity varied less than 1% over periods as long as 30 minutes. Apparently random variations in resistivity as large as 2.6% often occurred 6–8 hours after cortical exposure. The preparation was discarded when these variations became appreciable. No significant variation in cortical resistivity occurred when the anesthesia was deepened from a light pentobarbital level to a level of pentothal which induced electrical inactivity.

### *Spreading depression*

Figure 3 presents a series of records taken during a wave of spreading depression. Each trace shows the response to geniculate-radiation stimulation recorded from the pipettes across the cortex. Constant current pulses are applied before and during the specific response. All the pulses except those in trace (I) of this figure have been converted to positive pulses by the algebraic addition of a calibrated positive pulse taken from the phase splitter. The gain of the amplifier has been reduced in trace (I), and the full voltage pulse recorded by the pipettes is shown. An increase in resistivity of the cortex is indicated by the fall in the amplitude of the pulses, traces (A-H). The reduction of the specific response by the spreading depression is also shown. Both resistivity and response recover subsequently. That the electrical response evoked by the afferent stimulation has no measurable effect upon the cortical resistivity can be observed in the equal amplitudes of the early and late pulses. This was the case during all phases of the specific response even when it was exaggerated by strychnine applied to the pial surface.

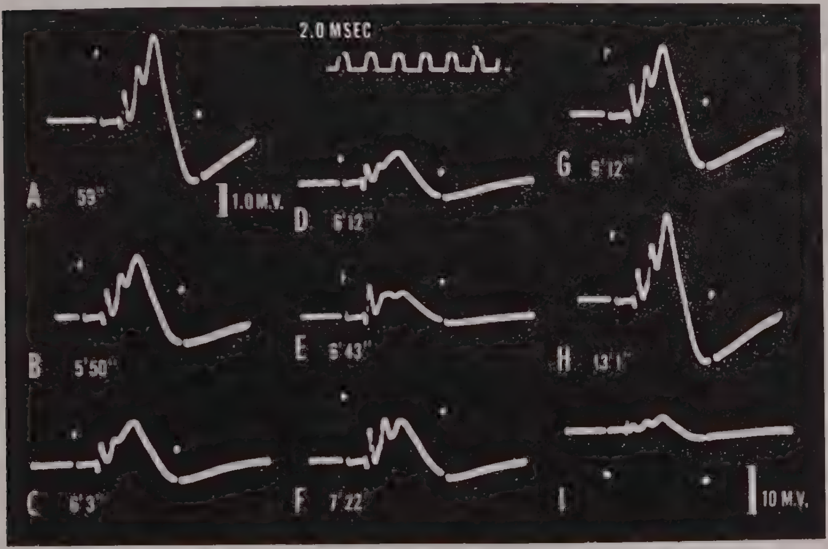

Fig. 3 Effect of spreading depression upon resistivity and response to subcortical stimulation. Time after the stimulus producing the depression is noted below each trace. Pulses for measurement of resistivity appear before and after application of a geniculate stimulus and are reduced by a calibrated opposing pulse in traces A-H. A decrease in pulse amplitude during the depression indicates a rise in resistivity. Surface positive deflections are upward in all traces. Trace I is at reduced gain, and the pulses are not opposed.

The time course of the depression in "spontaneous" electrical activity and the slower voltage variation of the surface and deeper structures in relation to a remote point have been

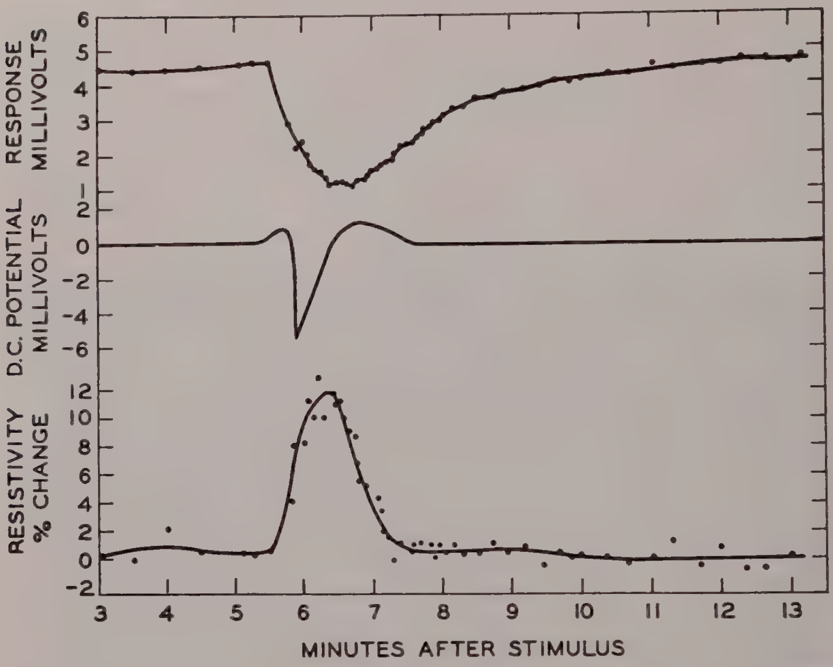

Fig. 4 Plot of total amplitude of specific response, slowly varying voltage across cortex, and percent resistivity change with time after initiation of a spreading depression. Same experiment as figure 3.

described by Leão ('44a, '51) and others.[2] The temporal relations of resistivity change, total amplitude of evoked response, and slowly changing voltage across the cortex in the experiment of figure 3 are plotted in figure 4. The peak increase in resistivity (deep electrode placed subcortically) lagged behind the peak surface negative voltage by a mean of 26 seconds, and fell to 50% at a mean of 32 seconds after the voltage had decayed to 50%.

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[2] In experiments where voltage was recorded between the pial surface and a subcortical point, the potential was a simple surface negative wave, sometimes followed by a small late positive drift. As the deep electrode was raised into the cortex, a surface negative-positive wave appeared even with the deep lead as close to the surface as .1–.4 mm. This early negativity indicates a propagation of the depression wave down from the surface as the wave progresses. We cannot confirm Graftein's ('54a) view that the prime sink is in cortical layer II. It should be noted that profound depressions may affect the response of radiation terminals in addition to intra-cortical structures. (First cortical spike, fig. 3).

For the 27 depressions in which the resistivity change was measured between the surface of the cortex and a deep point within or just below the lower border of the cortex, the mean resistivity increase was 13.0% ± 1.0 (S.E.). A decrease in resistivity was never observed with the electrodes placed across cortex alone. However, with the upper electrode placed at the gray-white junction and the deeper electrode placed within 1 mm below it, a decrease in resistivity was recorded. Confirmatory calculations of the resistivity change across this white matter were obtained from experiments where, with the superficial electrode at the surface, the deep electrode was initially placed in the white matter and, in subsequent depressions, raised into the cortex. This decrease in the white matter amounted to a mean of 5.2% ± 0.5 (S.E.) for 12 observations.

The cortical resistivity increase with spreading depression was greater in the more superficial than in the deeper layers. The mean change in the upper 0.8 mm of cortex was 23% ± 2 (S.E.) for 10 runs. In 5 experiments yielding data from which the per cent change could be calculated between depths 0.5 to 1.5 mm, the mean increase was 5.5% ± 0.7 (S.E.). Because of the possibility that a portion of the white matter was influencing the recorded field, an expression of a similar value for the change across the lower quarter of the cortex might be misleading; however, in some experiments large resistivity increases were measured across the deep layers.

The method described in figure 1 could not be adapted to provide parallel lines of current flow tangential to the pial surface. To measure tangential resistance, two platinum black electrodes were inserted to an equal depth in the cortex and connected to a Wheatstone bridge. The electrical changes were recorded between two pipettes placed across the cortex. An increase of similar magnitude to that recorded across the cortex in depth was obtained by this method.

The possibility that movement of the cortex by the vascular changes of spreading depression would alter the results must be considered. Strain gage measurements by Marshall ('53) have demonstrated a 5–20 μ maximum expansion of the cortex presumably produced by the vasodilatation described by Leão ('44b). Such a variation in cortical thickness could account for, at most, a 1% change in resistivity.

### *Direct cortical stimulation*

An electrical stimulus was delivered to the cortical surface from a triple electrode (tips 1 mm apart, one of which was a Wagner ground) about 1 mm from the site of the superficial pipette. The deeper electrode was located as close as possible to the junction of cortex and white matter. In figure 5, as in figure 3, the stimulus was delivered between the resistivity-measuring pulses; the slight rise in the amplitude of the second pulse above that of the first indicates a decrease in resistivity. The delay of the second pulse after the stimulus was varied during these experiments. The time course of the change in resistivity in one of the experiments is plotted in figure 6. In general, the course of the resistivity fall fol-

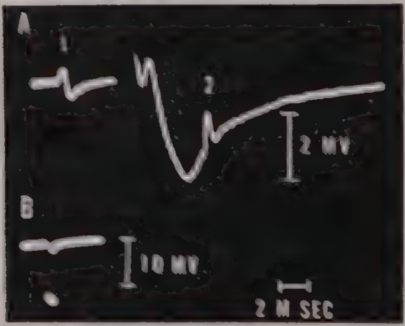

Fig. 5 Effect of direct cortical stimulation upon resistivity. In trace A the pulses for resistivity measurement (1 and 2) appear before and after the stimulus and are reduced by a calibrated opposing pulse. Increase in pulse amplitude indicates a 5% fall in resistivity. Trace B is at lower gain; the pulse is not opposed.

lows that of the surface negative response. The magnitude of the change shown in figures 5 and 6 is unusually large. The mean of the maximum decreases in resistivity in each of 20 observations was 3.1% ± 0.8 (S.E.).[3]

The possibilty was examined that measurements made with lines of current flow parallel to the pial surface might show a greater change upon direct cortical stimulation. The Wheatstone bridge technique referred to previously was employed to approximate this condition. No change could be detected, probably because of the insensitivity of this method.

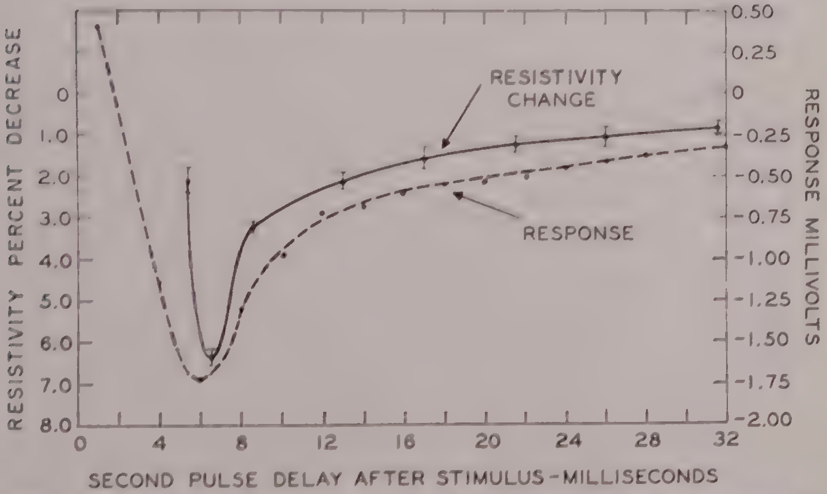

Fig. 6 Plot of percent resistivity change and voltage across the cortex with time after a direct stimulus. S. E. for 10 observations is indicated at each point.

## *Polarization*

For the polarization experiments, the two pipettes were placed across the cortex and between the polarizing electrodes. Polarizing current flow was adjusted to produce either

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[3] The effect of spreading depression upon the decrease in resistivity after direct stimulation was to reduce the fall by about half as the surface negative response was depressed.

50 or 100 mV of transcortical potential difference. Resistivity was measured in the manner described in figure 1.

A surface negative polarization of 50 mV produced a fall in resistivity of 1–3% (fig. 7). The change following the beginning of current flow usually required .25–2.0 seconds to reach a plateau, and recovery from polarization had a similar duration. With 100 mV, the resistivity change was usually greater; but the course during polarization was unsteady, and recovery to baseline was often incomplete. This suggested that permanent tissue changes were produced by the larger currents.

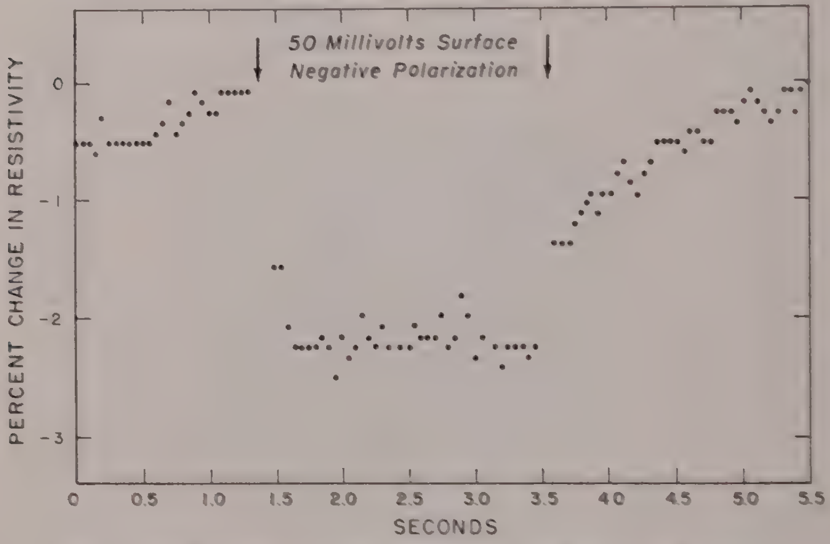

Fig. 7 Effect of 50 mV surface negative polarization across cortex.

Surface positive polarization at either strength produced no consistent result, even in observations repeated at the same point.

## DISCUSSION

Active peripheral nerve has been shown to have a many fold decrease in membrane resistance when conduction is all-or-none. A comparable reduction in the mean resistivity of the neuronal membranes in the cortex with electrical activity seems unlikely. The total membrane area of cortical neurones is primarily dendritic, and it is doubtful whether the major portion of dendritic membrane conducts propagated action potentials, for there is suggestive evidence that dendrites usually depolarize electrotonically. The same structural consideration implies that the resistivity measurements of this study are largely influenced by membrane resistance changes of dentrites.

The true magnitude of the change in neuronal membrane resistivity cannot be estimated from these data since the effect of other resistances is unknown. Furthermore, if one considers the configuration of intracortical structures and of interstitial space, the hypothesis that the membrane potential of a part of a cortical neurone varies with the depth of that part can scarcely be rejected by failure to detect a consistent difference in resistivity between the superficial and the deep portions of unstimulated cortex.

That spreading depression produced an increase in resistivity which was at least twice the magnitude of the decrease produced by direct stimulation is remarkable. Alone, the cessation of neuronal activity with spreading depression might raise the resistivity of the cortex, but the administration of pentothal until the complete suppression of spontaneous activity did not significantly alter resistivity.

The negativity of the more superficial cortical layers with respect to the deeper ones during spreading depression suggests either a depolarization of the superficial parts of the cortical neurones or a hyperpolarization of their deeper portions. If the membrane potentials in the deeper structures were increased, the resistivity of the cortex might be expected to rise; however, it was in the more superficial layers that the greatest increase occurred. Moreover, that the resistivity of the subcortical white matter was reduced implies that the white matter was being electrotonically depolarized, since there is no evidence that it is an active participant in spreading depression. Such contradictory results lead one to question the value of the model of peripheral nerve in cortical physiology unless spreading depression increases resistivity in a way not included in the model.

The assumption may be questioned that the increase in cortical resistivity with spreading depression is a measure of neuronal membrane resistivity. Leão and Martins Ferreira ('53) have produced an increase in resistivity by anoxia; the increase was comparable in magnitude to that observed during spreading depression.[4] Similar results were obtained in this study. It may be that the disturbances of this phenomenon produce alterations of glial, as well as neural, structures. For example, a cellular swelling would increase the resistance of the extracellular current shunt, thus increasing the tissue resistance. The apparently conflicting results of spreading depression may be reconciled by supposing that the net change in resistivity is a combination of effects such as a depolarization of the neural structures in the cortex, and another process such as a swelling of cells. The dissimilarity in time course of slow voltage variation and resistivity rise support this view.

The cortical resistivity was measurably decreased during the response to direct stimulation, but unaffected by responses of comparable amplitude elicited by afferent stimulation and strychnine. This contrast may relate to a difference in the degree of synchronization of activity. It may also indicate that gross potential recordings from the cortex activated by different routes are not validly comparable measures of the number of active cortical neurones, or of the nature of their activity.

The effect of polarizing current upon resistivity was of the same order as that produced by direct stimulation. The absence of an effect of surface positive polarization comparable to that of surface negative polarization may be the result of rectification similar to that observed in the squid axon (Cole and Baker, '41; Cole and Curtis, '41).

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[4] It is not likely that anoxia is causative in spreading depression since Marshall ('53) has demonstrated polarographically that the development of spreading depression is independent of the local oxygen tension.

Further conclusions would require more direct measures of neural membrane resistivity, possibly with ultramicropipettes.

## SUMMARY

1. A method for the simultaneous measurement of cerebral cortical resistivity and electrical activity is described. The resistivity is measured by the potential difference produced across the cortex by a constant current pulse. The method is sensitive to changes of 1%.

2. The resistivity of cortex under normal conditions is stable to within 1% and the mean is 222 ohm cm.

3. Spreading depression produces a 10–20% increase in total cortical resistivity most marked in the superficial layers, and a decrease in the subjacent white matter of about 5%.

4. Cortical resistivity is unaffected by changes in the level of cortical activity ranging from that of severe narcotization to that of maximal afferent stimulation under light anesthesia.

5. Direct cortical stimulation produces decreases in resistivity of about 3%.

6. Surface negative polarization also decreases resistivity 1–3%.

## ACKNOWLEDGMENTS

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# INHIBITORY EFFECTS OF SUGARS ON THE GROWTH OF THE MEALWORM, TENEBRIO MOLITOR L.[1]

G. FRAENKEL

*Department of Entomology, University of Illinois, Urbana, Illinois*

It is well known that the chief function of carbohydrates in the diet of animals is that of providing an easily available source of energy. Many investigations show that a number of carbohydrates, such as starch, glycogen, dextrin, maltose, sucrose, glucose and fructose can be freely substituted for each other in the food of an animal. Insects, however, seem to be the only group in which the effects of many different carbohydrates on growth and survival have been studied systematically. The results have been especially striking and significant in the case of flying insects which normally are dependent on the nectar of flowers as a readily available source of energy. The life span of some of the more rapidly flying diptera and hymenoptera, for example, may be increased from a few days to many weeks merely by feeding a suitable sugar to the newly emerged adult insects. This phenomenon has been utilized to investigate the food value of a great number of carbohydrates in the metabolism of flies (Haslinger, '35; Fraenkel, '36, '40; Hassett, '48; Hassett et al., '50), and bees (Vogel, '31).

More recently, the effects of different carbohydrates in the nutrition of growing beetle larvae have been investigated for *Tenebrio molitor* (Leclercq, '48), *Tribolium confusum* (Bernard and Lemonde, '49), *Stegobium paniceum* and *Orycaephilus surinamensis* (Lemonde and Bernard, '53). From the

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results of feeding tests various carbohydrates have been classified according to their ability to sustain life or promote growth of an insect. With rare exceptions, however, such as the report on the toxicity of mannose to the honey bee (Vogel, '31) it has not been recognized that sugars may actually cause inhibitory effects on the development of the insects which feed upon them. The present investigation forms part of a comprehensive study of the nutritional requirements of the mealworm larva, *Tenebrio molitor,* which normally lives on cereal seeds and requires about 80% carbohydrates in the diet. When the effects of different carbohydrates on growth and survival were determined, it was found that many of them produced strong inhibitory effects. Although toxic effects of galactose on various organisms have previously been observed, no comparable study of the inhibitory actions of sugars seems to have been reported for any organism.

## METHODS

The methods of growing *Tenebrio larvae* on semi-synthetic diets have already been described (Fraenkel, Blewett and Coles, '50; Fraenkel, '51). The diet consisted of 80 parts glucose or starch, 20 parts casein (Labco), 1 part cholesterol, 2 parts McCollum's salt mixture no. 185 and the following vitamins of the B-complex, expressed as microgram per gram of the dry diet: thiamin 25, riboflavin 12.5, nicotinic acid 50, pyridoxin 12.5, pantothenic acid 25, choline chloride 500, i-inositol 250, biotin 0.25, folic acid 2.5 and carnitine from 0.75 to 3.00 μg. Previous to the discovery of carnitine as a necessary growth factor for *Tenebrio* (Carter et al., '52), the diet had to be supplemented with 2% yeast or another suitable source of carnitine. When the experiments were started (about 4 years ago) tests with many different sugars were run and included a supplement of either carnitine or 2% yeast in the diet. Essentially the same results were obtained with either substance. More recently a situation has arisen in which *Tenebrio* larvae no longer grow on the basic diet in the presence of carnitine, unless the diet also contains yeast or some other chemically undefined preparation. In order to exclude the possibility of any untoward effects which might be due to factors other than those to be investigated in the present study all the experiments to be reported refer to diets supplemented with 2% dried debittered brewers yeast.

The experiments were performed in 1 oz. wide mouth bottles, each containing 3 gm of food and 10 larvae, and each test was run in duplicate, i.e. with 20 larvae. Experiments were started with newly emerged larvae and, as a rule, terminated after 8, 9 or 10 weeks. At this time the larvae on a good diet reached a weight of 50 to 100 mg. The results have been tabulated in a manner to show the number of larvae surviving and their average weight after the feeding period. Each table represents a series of tests run simultaneously, except for table 1, which combines data from 4 tests in which the weights on the positive control diet (starch) were virtually identical.

## EXPERIMENTS

Table 1 shows the results of feeding experiments with 24 different carbohydrates fed at the 80% level. The larvae grew well on glucose, maltose, dextrin, starch and glycogen, and moderately well on mannitol, lactose, melibiose, trehalose, cellobiose, raffinose and melezitose. In the present experiments contrary to earlier findings (Fraenkel et al., '50), the larvae always grew significantly faster in the presence of starch than of glucose. On sucrose the larvae grew well for 7 weeks, after which time the diets became wet and the larvae died. This happened every time sucrose was tested, and mostly after shorter periods of growth. The larvae failed to grow, or grew very little on arabinose, xylose, galactose, sorbose, inulin, cellulose and α-methylglucoside. On fructose, mannose, sorbitol and dulcitol the larvae died very soon. Subsequent tests revealed that the larvae also failed to grow on other pentoses namely rhamnose and ribose, and on α-methylmannoside. The effect of lactose proved to be variable and on most occasions larvae grew very slowly, as for instance in the tests of table 2.

It was obvious in the case of fructose, mannose, sucrose and sorbitol, that the diets were hygroscopic and became too wet for *Tenebrio* larvae. Subsequently the sugars which had been shown to be of poor nutritional value at the 80% level were offered mixed with equal parts of starch. It was argued that the larvae failed to grow on a particular carbohydrate for three different reasons.

TABLE 1

*Tenebrio molitor on diets containing 80% carbohydrate. The number of larvae surviving and average weight after 9 weeks*

| | NUMBERS | WEIGHT | | NUMBERS | WEIGHT |
|---|---|---|---|---|---|
| | | *mg* | | | *mg* |
| Arabinose | 8 | 1.5 | Lactose | 17 | 20.9 |
| Xylose | 9 | 1.2 | Cellobiose | 16 | 27.3 |
| Glucose | 20 | 51.2 | Trehalose | 18 | 25.6 |
| Fructose [1] | died soon | | Melibiose | 19 | 24.0 |
| Galactose | 6 | 4.3 | Raffinose | 17 | 38.2 |
| Mannose [1] | died soon | | Melezitose | 18 | 35.1 |
| Sorbose | 1 | 1.0 | Dextrin | 20 | 68.7 |
| Mannitol | 12 | 20.1 | Starch | 16 | 73.7 |
| Sorbitol [1] | died soon | | Glycogen | 19 | 79.6 |
| Dulcitol | died soon | | Inulin | 7 | 5.3 |
| Sucrose [2] | 20 | 17.1 | Cellulose | 20 | 2.7 |
| Maltose | 13 | 48.3 | α-methyl-glucoside | 13 | 1.6 |

[1] Diet wet.

[2] At 7 weeks; diet subsequently became wet and larvae died.

1. The carbohydrates could be nutritionally adequate but render the medium too wet for the larvae to exist.
2. The carbohydrate could be inert in the sense that the larvae were unable to utilize it for energy.
3. The carbohydrate might be outright toxic.

In the first case it was assumed that the starch would take up the excess moisture. Should the mixture of a particular carbohydrate with starch then prove to be as good as starch alone, a good nutritional value of the sugar would be indicated. The use of presumably inert fillers afforded a means of investigating reasons 2 and 3. In the second case, a carbohydrate could be regarded as inert if, after mixing with starch, growth proceeded at the same rate as on a starch-cellulose or starch-glass powder ration. Finally, should the mixture be inferior to the starch + inert filler diet, the sugar would have been shown to be inhibitory.

TABLE 2

*Tenebrio molitor on diets containing 80% carbohydrate or mixtures of equal parts of a carbohydrate and starch. Numbers of larvae surviving and weight after 9 weeks*

| | 80% CARBOHYDRATE | | 40% PLUS 40% STARCH | |
|---|---|---|---|---|
| | Nos. | Weight | Nos. | Weight |
| | | *mg* | | *mg* |
| | Experiment 700 | | | |
| Sucrose | 13 | 5.0 | 19 | 83.6 |
| Sorbitol | 5 | 1.0 | 18 | 102.0 |
| Cellulose | 20 | 2.7 | 20 | 48.7 |
| Inulin | died soon | | 17 | 35.7 |
| Starch | 18 | 84.7 | | |
| | Experiment 712 | | | |
| Cellobiose | 17 | 24.3 | 19 | 91.3 |
| Lactose | 18 | 8.6 | 18 | 100.4 |
| Starch | 19 | 104.7 | | |
| Cellulose | | | 20 | 64.7 |
| Glass | | | 20 | 68.8 |

Table 2 shows clearly that the mixture of either 40% sucrose or 40% sorbitol with equal quantities of starch was as good, if not better than starch alone. A similar result had ben obtained earlier with fructose. We may therefore conclude that these three substances have excellent nutritional value once the excess moisture has been eliminated. The effect of lactose or cellobiose in combination with starch was as good as that of undiluted starch. It can also be seen that cellulose serves as a nutritionally inert material in these studies, since it has the same effect in the diet as glass powder (a preparation of very small spherical particles).

Table 3 shows that in all the other cases the larvae either failed to grow on diets containing equal parts of starch and diverse carbohydrates, e.g. rhamnose, sorbose, α-methylglucoside or dulcitol, or grew much slower than on the starch cellulose mixture, as in the case of arabinose, xylose, galactose and mannose. With the exception of galactose, inhibitory effects were manifest with these sugars at the 20% level. The 40%–40% mixture of test sugars with glucose showed even greater inhibition of growth than with starch.

TABLE 3

*Growth of Tenebrio larvae on diets containing mixtures of starch or glucose with other carbohydrates in proportions of 40% – 40% or 60% – 20%. Number of larvae surviving and average weights after 9 weeks*

| | 40% + 40% STARCH | | 20% + 60% STARCH | | 40% + 40% GLUCOSE | |
|---|---|---|---|---|---|---|
| | Nos. | Weight | Nos. | Weight | Nos. | Weight |
| | | *mg* | | *mg* | | *mg* |
| Arabinose | 20 | 15.4 | 20 | 15.9 | 19 | 15.6 |
| Rhamnose | 17 | 2.9 | 20 | 9.6 | 18 | 4.0 |
| Xylose | 20 | 14.7 | 19 | 17.4 | 19 | 15.7 |
| Galactose | 18 | 21.0 | 19 | 69.5 | 12 | 7.6 |
| Mannose | 19 | 16.0 | 20 | 46.0 | 0 | . . . |
| Sorbose | 7 | 2.3 | 16 | 10.0 | 2 | 1.5 |
| Inulin | 20 | 55.8 | 20 | 74.9 | 19 | 19.9 |
| Cellulose | 19 | 65.7 | 18 | 93.1 | 20 | 35.0 |
| α-methylglucoside | 12 | 1.5 | 20 | 6.7 | 0 | . . . |
| Dulcitol | 0 | . . . | 12 | 6.3 | 0 | . . . |
| 80% starch | 19 | 73.8 | | | | |
| 80% glucose | 18 | 48.0 | | | | |

The effect of cellulose in equal proportions with starch or glucose was somewhat variable. In the experiments of table 2 growth was slower than on starch or glucose alone, while in those of table 2 it was almost as good, and seemed even better on the 20/60% ratio. It is, however, quite clear from tests performed on various occasions that on the 20% level or below the admixture of cellulose or another inert filler has no effect on the growth rate of *Tenebrio.*

Having established the inhibitory effects of a number of different carbohydrates, the proportion of a particular substance to starch or glucose was decreased in order to determine the threshold concentrations of inhibition for various sugars. Table 4 shows the results with the pentoses arabinose, xylose, rhamnose and ribose. Arabinose shows inhibitory effects at the 20% level with starch and at 5 or 2% with glucose; xylose at 10% with starch and 2% with glucose; rhamnose at 10% with starch and 5% with glucose; and ribose at the unusually low level of 2% with starch and glucose.

TABLE 4

*Tenebrio molitor on mixtures of pentoses and starch or glucose (80% total carbohydrate). Number surviving and average weight after 9 weeks*

| | | PLUS STARCH | | PLUS GLUCOSE | |
|---|---|---|---|---|---|
| | | Nos. | Weight | Nos. | Weight |
| | % | | mg | | mg |
| Arabinose | 20 | 11 | 49.1 | 19 | 16.5 |
| | 10 | 18 | 73.0 | 20 | 24.6 |
| | 5 | 19 | 80.4 | 20 | 24.5 |
| | 2 | 18 | 80.2 | 20 | 28.2 |
| Xylose | 20 | 15 | 55.7 | 20 | 19.5 |
| | 10 | 19 | 65.0 | 17 | 22.0 |
| | 5 | 19 | 86.3 | 20 | 17.2 |
| | 2 | 16 | 86.7 | 19 | 22.3 |
| Rhamnose | 20 | 18 | 36.2 | 20 | 8.6 |
| | 10 | 18 | 60.6 | 20 | 14.2 |
| | 5 | 19 | 73.0 | 20 | 16.0 |
| | 2 | 18 | 86.8 | 19 | 28.4 |
| Ribose | 20 | 19 | 6.8 | 0[1] | ... |
| | 10 | 13 | 10.3 | 0[1] | ... |
| | 5 | 17 | 40.3 | 17 | 6.3 |
| | 2 | 18 | 58.1 | 20 | 11.8 |
| Starch | 80 | 19 | 96.6 | | |
| Glucose | 80 | | | 19 | 36.6 |

[1] Diet too wet.

Table 5 shows corresponding results with the hexoses galactose, mannose and sorbose. Galactose proved to be inhibitory at the 20% level with starch and at 10% with glucose; mannose at the 10% level with starch and the 5% level with glucose, and sorbose at the 5% level with both starch and glucose.

Some of the strongest inhibitory effects were shown with the sugar alcohol dulcitol and with α-methylglucoside (table 6). Dulcitol showed strikingly toxic effects at the 2% level with glucose, and α-methylglucoside at 1% with either starch or glucose. α-Methylmannoside inhibited growth at the 10% level with starch and the 2% level with glucose.

TABLE 5

*Tenebrio molitor on mixtures of galactose, mannose or sorbose with starch or glucose in varying proportions (80% total carbohydrate). Number of larvae surviving and average weight after 9 weeks*

| | | SUGAR PLUS STARCH | | | SUGAR PLUS GLUCOSE | |
|---|---|---|---|---|---|---|
| | | Nos. | Weight | | Nos. | Weight |
| | % | | mg | % | | mg |
| Galactose | 40 | 16 | 8.5 | 20 | 18 | 9.6 |
| | 20 | 20 | 32.7 | 10 | 19 | 23.3 |
| | 10 | 19 | 54.4 | 5 | 20 | 28.9 |
| + uridine .1 or 1% | 40 | 19 | 8.3 | 2[1] | 20 | 27.9 |
| Mannose | 20 | 20 | 16.1 | 20 | diet too wet, died | |
| | 10 | 19 | 35.7 | 10 | 7 | 13.2 |
| | | | | 5 | 16 | 11.6 |
| | | | | 2 | 20 | 28.1 |
| Sorbose | 10 | 17 | 34.2 | 10 | 14 | 10.3 |
| | 5 | 20 | 39.6 | 5 | 20 | 20.4 |
| | 2 | 20 | 51.6 | 2 | 19 | 34.4 |
| | 1 | 20 | 59.8 | 1 | 20 | 29.9 |
| Starch | 80 | 20 | 59.1 | | | |
| Glucose | 80 | | | | 20 | 31.3 |

[1] No addition of uridine.

Table 7 gives a summary of the smallest proportion of a given carbohydrate required to show an inhibitory effect on the growth of *Tenebrio*. It is evident that the toxicity was enhanced by the presence of glucose in the ration.

TABLE 6

*Tenebrio molitor on mixtures of dulcitol, α-methylglucoside or α-methylmannoside with starch or glucose in varying proportions (80% total carbohydrate). Number of larvae surviving and average weight after 8 weeks*

| | | PLUS STARCH | | PLUS GLUCOSE | |
|---|---|---|---|---|---|
| | | Nos. | Weight | Nos. | Weight |
| | % | | | | |
| Dulcitol | 10 | 20 | 49.0 | 9 | 1.0 |
| Dulcitol | 5 | 20 | 36.8 | 17 | 7.1 |
| Dulcitol | 2 | 20 | 63.3 | 19 | 17.9 |
| Dulcitol | 1 | 18 | 67.7 | 19 | 27.8 |
| α-methylglucoside | 10 | 20 | 13.2 | 19 | 3.6 |
| α-methylglucoside | 5 | 20 | 31.1 | 18 | 7.4 |
| α-methylglucoside | 2 | 19 | 38.6 | 19 | 24.8 |
| α-methylglucoside | 1 | 17 | 43.4 | 19 | 23.5 |
| α-methylmannoside | 40 | 20 | 34.7 | 20 | 12.5 |
| α-methylmannoside | 20 | 19 | 40.6 | 20 | 10.1 |
| α-methylmannoside | 10 | 19 | 43.5 | 19 | 12.6 |
| α-methylmannoside | 5 | | | 20 | 14.2 |
| α-methylmannoside | 2 | | | 20 | 23.8 |
| Starch | 80 | 19 | 80.3 | | |
| Glucose | 80 | | | 20 | 35.6 |

TABLE 7

*Smallest inhibitory levels of certain sugars in the diet of Tenebrio molitor. The sugars were mixed with starch or glucose at a total level of 80% carbohydrates*

| | WITH STARCH | WITH GLUCOSE |
|---|---|---|
| | % | % |
| Arabinose | 10–20 | 5 |
| Xylose | 10 | 2 |
| Rhamnose | 10 | 5 |
| Ribose | 2 | 2 |
| Galactose | 20 | 10 |
| Mannose | 10 | 5 |
| Sorbose | 5 | 5 |
| Lactose | 40 | |
| α-methylglucoside | 1 | 1 |
| α-methylmannoside | 10 | 2 |
| Dulcitol | 5 | 2 |

It was considered of interest to see if the results of the feeding tests could be correlated with the presence of the appropriate digestive enzyme in the intestine of the larvae. Several intestines were ground up with sand and the resulting homogenate incubated under toluene for two days with a few drops of a 10% solution of a carbohydrate. The formation of glucose or fructose from a di- or trisaccharide was determined by the osazone test. With the exception of melibiose, this method demonstrated the presence of a hydrolyzing enzyme in every single case where good or fair growth had taken place (see table 8). Lactase was demonstrated on two out of 5 determinations. This result was in line with the great variability of the effect of lactose in the feeding tests reported earlier. α-Methylglucoside which was of no nutritional value for *Tenebrio* larvae was not hydrolyzed. It should be mentioned that in similar tests α-methylglucoside was hydrolysed by an enzyme extract from the intestine of a fly, *Sarcophaga* sp., which has been shown to utilize this substance for survival. The tests with the fly were performed

TABLE 8

*The presence of carbohydrase in the intestine of Tenebrio molitor, tested with one osazone method. The formation of glucosazone crystals was considered indicative of the presence of the enzyme. The third column indicates the nutritional value of the sugars as shown by feeding tests*

| SUGAR TESTED | FORMATION OF GLUCOSAZONE CRYSTALS | NUTRITIONAL VALUE |
|---|---|---|
| Sucrose | + | + + + |
| Maltose | + | + + + |
| Lactose | ± | + |
| Cellobiose | + | + + |
| Turanose [1] | + | |
| Melibiose | — | + + |
| Trehalose | + | + + |
| Raffinose | + | + + |
| Melezitose | + | + + |
| α-methylglucoside | — | — |
| Starch | + | + + + |

[1] Turanose arises in the hydrolysis of melezitose.

as a check on the method employed for the determination of α-glucosidase activity.

## DISCUSSION

In considering the results, one must distinguish clearly between phenomena which might involve the action of hydrolytic enzymes and others where this consideration would not apply. In the case of pentoses, hexoses and sugar alcohols one would expect absorption to take place directly from the intestine, while disaccharides, trisaccharides, polysaccharides and glucosides would require previous cleavage to their constituent monosaccharides. Most of the results with di-, tri- or polysaccharides can be interpreted on the assumption that the corresponding carbohydrases are either present, absent, or present in suboptimal amounts and that galactose (which arises in the cleavage of some of the toxic sugars) had a specific inhibitory effect.

The inferior effect of melibiose, lactose and raffinose could be, in part, attributed to the formation, during digestion, of galactose, which by itself and especially in combination with glucose has a strong inhibitory action. The presence of the appropriate enzyme was demonstrated qualitatively in *in vitro* determinations in every single case of successful growth, with the exception of melibiose. The presence of melibiase has to be postulated from the feeding tests inspite of the fact that it was not demonstrated *in vitro*. In the case of lactose the results of both feeding and enzyme tests were variable which may indicate lactase to be present at a relatively low level. It is interesting to note that the nutritional value of melibiose was significantly higher than that of a glucose-galactose mixture which would have been the result of hydrolysis, and the same phenomenon was observed in some tests with lactose.

The presence of cellobiase, demonstrated both directly in enzyme tests and indirectly in feeding tests, and the indication for the presence of lactase were unexpected since β-glycosidases have rarely been demonstrated in insects. β-

Glucosidase has, however, been recently reported for the cockroach (Newcomer, '54). The failure to utilize α-methylglucoside was also somewhat unexpected since this glucoside has previously been shown to be utilized by flies (Fraenkel, '40) and the bee (Vogel, '31). It is worth recording that the maltase of *Tenebrio* does not apparently act on α-methylglucoside.

In the case of the pentoses, hexoses and dulcitol, it is considered improbable that their inhibitory effects were the result of a failure to be absorbed from the intestine. The possibility remains that after absorption these sugars do not enter into the metabolic scheme of the larva either via conversion to glucose or by a direct oxidative route. Galactose, for instance, might not be utilized in the absence of an uridine-linked enzyme system which mediates the conversion of galactose to glucose (Caputto et al., '50). However, the nutritive value of a diet which contained galactose was not improved by the addition of uridine (see table 4). In any case, a block of absorption or oxidation would not readily explain why the presence of an inhibitory sugar prevented the utilization of from 10 to 50 times larger quantities of glucose or starch.

Considering the manifold differences in structure of the inhibitory sugars, and the close similarity in the order of magnitude of inhibitory effects it is felt that a common explanation might account for all the effects observed. The two possibilities regarding sites of inhibition are either (a) at the intestinal wall or (b) after absorption from the intestine. In the first case, the inhibitory sugar might conceivably interfere with the absorption of glucose from the intestine. This could be the case with α-methylglucoside which does not undergo hydrolysis in the intestine and could therefore be considered to exert its effect in the lumen of the intestine. In the second case, it appears possible that adverse effects are due to competitive inhibition of glycolytic processes.

Inhibitory effects of many different sugars, as described here, do not seem to have been recorded before for any organism. Apparently, insects have been the only organisms for which the nutritional effects of many carbohydrates have been systematically studied. Specific inhibitory effects of carbohydrates have not been described before with insects except for evidence on toxic effects of mannose on the bee (Vogel, '31). This would not seem surprising in studies with adult flies or hymenoptera where the duration of life in the absence of a sugar is only two to three days. Such a short life span would obscure any inhibitory effect. The extensive feeding experiments of Bernard and Lemonde ('49) with *Tribolium confusum* and Lemonde and Bernard ('53) with *Stegobium paniceum* and *Orycaephilus surinamensis* do not contain data on development in the absence of a carbohydrate and do not take into account the possibility of physical factors such as excessive moisture. It is, however, well known (Fraenkel and Blewett, '43) that *Tribolium* can reach the pupal stage, although at a lower rate of growth, in the complete absence of a carbohydrate. The failure of *Tribolium* to develop on xylose, arabinose or galactose (Bernard and Lemonde, '49) can therefore be attributed to the inhibitory effect of these three sugars. This interpretation cannot be applied to the two other beetles studied by the same authors, because, according to Fraenkel and Blewett ('43), they do not grow in the absence of a carbohydrate. The effects of certain sugars which were inhibitory for *Tenebrio,* were strikingly different for these two insects. *Orycaephilus* failed to grow on the 4 pentoses while *Stegobium* grew on all but ribose. *Orycaephilus* failed to grow on sorbose, dulcitol and inulin, and *Stegobium* on galactose, mannose and cellobiose. Neither developed on $\alpha$-methylglucoside. It is, however, not possible from these data to conclude which of these sugars were merely inert and which harmful.

Previous work on the utilization of carbohydrates by *Tenebrio* (Leclercq, '48) had shown that arabinose, galactose, and sorbose were of little value while the larvae grew well on glucose, maltose, lactose, raffinose, glycogen and starch, and to some extent on xylose. However, the tests were started with larvae of 35 mg which had grown on a natural diet for 50 days. We have observed that combinations of starch with certain sugars (including xylose) which are harmful to first stage larvae, can be utilized to some extent by older larvae. Thus, Leclercq's data also do not permit classification of carbohydrates as either inert or harmful.

No systematic investigations seem to exist on the nutritional value of many different carbohydrates for chicks, rats or other vertebrates. However, harmful effects of galactose and lactose have ben freqently described (e.g. Guha, '31; Mitchell and Dodge, '35), and have recently acquired new interest since the discovery of the role of uridine diphosphate in the enzymatic conversion of galactose into glucose (Caputto et al., '50; Rutter and Hansen, '53). Of some relevance are the experiments of Harris and Kutsky ('53) on the utilization of added sugars by chick heart fibroblasts. Utilized were d-glucose, d-mannose, d-maltose and glycogen. Not utilized were l-glucose, d-galactose, d-sucrose, d-lactose, d-ribose, d-arabinose, d-xylose, d-erythrose and raffinose. All the non-utilized sugars were completely inert except galactose which was definitely harmful.

## SUMMARY

1. The effect of 27 different carbohydrates on the larval development of the mealworm, *Tenebrio molitor,* was studied. Development was good on glucose, maltose, dextrin, starch and glycogen, fairly good on mannitol, cellobiose, trehalose, melibiose, melezitose and raffinose and variable on sucrose and lactose. The larvae failed to grow on arabinose, xylose, rhamnose, ribose, fructose, mannose, galactose, sorbose, sorbitol, dulcitol, inulin, cellulose, α-methylglucoside and α-methylmannoside.

2. The adverse effects of fructose, sorbitol, and in some cases sucrose, were solely caused by their strong hygroscopic properties. Cellulose was entirely inert.

3. The presence of the appropriate hydrolysing enzyme has been demonstrated for sucrose, maltose, cellobiose, tura-

nose, trehalose, raffinose, melezitose and starch, and in some instances, lactose. It has not been demonstrated for melibiose or α-methylglucoside.

4. Tests with mixtures of various carbohydrates and either starch or glucose showed that all the other adverse sugars exerted a strong inhibitory effect on development of *Tenebrio*. When the diets contained 80% carbohydrate, these effects were exhibited at levels of 1 to 10% of the ration, according to the carbohydrate employed. The possible nature of these inhibitory effects is discussed.

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# AN ENZYMATIC COMPARISON OF TWO INTERBREEDING BUT GENETICALLY ISOLATED VARIETIES OF PARAMECIUM AURELIA [1,2]

MYRON LEVINE [3]
*The McCollum-Pratt Institute, The Johns Hopkins University,*
*Baltimore 18, Maryland*

The ciliated protozoan, *Paramecium aurelia,* can be divided into eight physiological species or varieties, each genetically isolated from the other. The mixing together of cultures of the complementary mating types of these varieties generally results in weak mating reactions, if they occur at all; and when they lead to conjugation, generally gives rise to high Fl viability. Exceptions to this rule are the interactions of the stocks of varieties 4 and 8. Under optimum conditions, these intervarietal mating reactions are as strong as those occurring within a variety, yet the Fl generation is almost completely nonviable (Sonneborn and Dippell, '46a and b; Sonneborn, '47 and '50; Levine, '53).

The mechanism, on the biological level, maintaining the genetic isolation of varieties 4 and 8 was recently studied by Levine ('53). Death of these intervarietal hybrids is due to a two way nucleo-cytoplasmic incompatibility system. Small amounts of cytoplasm of each variety are antagonistic to the normal development and functioning of the nuclei of

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[1] Contribution No. 97 of the McCollum-Pratt Institute.

[2] The following abbreviations will be used: DPN and TPN, diphosphopyridine nucleotide and triphosphopyridine nucleotide respectively; DPNH and TPNH, reduced diphosphopyridine nucleotide and reduced triphosphopyridine nucleotide respectively; DPNase, diphosphopyridine nucleotidase; ATP, adenosine triphosphate; ATPase, adenosine triphosphatase.

[3] Postdoctoral Fellow of the American Cancer Society. Present address: Department of Bacteriology, University of Illinois, Urbana, Illinois.

the other variety; nuclear material is actually destroyed in the extreme case.

An understanding of this nucleo-cytoplasmic incompatibility on the biochemical level would be of prime interest. However, little is known of the metabolism and biochemistry of these organisms. In an effort to learn something of the metabolism of paramecia and in an effort to learn to handle these animals biochemically an enzymatic comparison of varieties 4 and 8 was started. It was hoped that some clues to the biochemistry of the lethal interaction might become evident. This paper presents the results of the enzymatic comparison and describes some preliminary observations on the lethal interaction.

## MATERIALS AND METHODS

Stock 51 sensitives, mating type VIII of variety 4 and stock 131 sensitives, mating type XV of variety 8, the two stocks used most extensively in establishing the nucleo-cytoplasmic incompatibility system, are the paramecia used for this work. The cultures were grown in three and four liter flasks on a Ceraphyl infusion inoculated with *Aerobacter aerogenes,* according to the methods of Sonneborn ('50). Their effective average growth rate was less than one fission per day. In preparation for assay, cultures last fed two days previously were harvested in Berkefeld filters, further concentrated by low-speed centrifugation, and washed several times in exhausted autoclaved culture fluid. These concentrated cultures were allowed to stand for approximately two hours in this medium and were again washed. This procedure removed the bacteria as a contributory factor to the enzyme assays. Finally, the paramecia were made into a brei in the appropriate buffer by drawing them in and out of a hypodermic syringe several times. Between four and twelve million animals were handled in this manner for a single day's assays.

The assays to be reported on are with one exception either colorimetric or spectrophotometric and were carried out on whole breis. The specific activities given are the highest ones obtained and since considerable variation in activity was found for some enzyme assays and since few attempts were made to find optimum conditions, the quantitative aspects of the study are not stressed. All specific activities are calculated as per milligram protein per unit of time. Protein concentration was estimated according to Lowry et al. ('51).

Phosphohexoisomerase was detected by the conversion of fructose – 6 – phosphate to glucose –6 – phosphate in the pressence of the brei. The disappearance of fructose – 6 – phosphate incubated for 60 minutes at 37° C. was measured by the method of Roe ('34). The assay for the enzymatic formation of desoxyribose – 5 – phosphate (desoxyribose – 5 – phosphate aldolase) was carried out according to Racker ('52). 2, 3, and 5 nucleotidase activities were followed by the release of inorganic phosphate from these adenylic nucleotides. Inorganic phosphate was measured according to Fiske and Subbarow ('25).

Except for ATPase activity all the other enzymes were assayed spectrophotometrically in the Beckman DU spectrophotometer by observing the change in optical density at the appropriate wavelength. The unit of enzyme activity for the following enzymes is defined as the change in log $Io/I_1$ of 0.001 per unit time per milligram protein.

Triose phosphate dehydrogenase, aldolase (Cori et al., '48), lactic dehydrogenase (Racker, '52), glycerophosphate dehydrogenase (Racker, '47), alcohol dehydrogenase (Racker, '50), glucose – 6 – phosphate dehydrogenase (Kornberg, '50), 6– phospho-gluconic dehydrogenase (Horecker and Smyrniotis, '50) and isocitric dehydrogenase (Grafflin and Ochoa, '50) were assayed by observing the change in optical density at 340 mμ; the change being due to the reduction of DPN or TPN and the oxidation of DPNH or TPNH.

Hexokinase activity was measured by coupling to the action of an exogenous source of glucose – 6 – phosphate dehydrogenase (prepared by Dr. R. D. De Moss from *Leuconostoc*). Glucose – 6 – phosphate produced from the action of hexokinase on glucose and ATP becomes available for the reduction of TPN by the glucose – 6 – phosphate dehydrogenase. By starting the reaction with citrate and coupling to the isocitric dehydrogenase found in paramecia aconitase activity could be detected. Attempts were made to detect the cleavage of ribose – 5 – phosphate or ribulose – 5 – phosphate into 3 and 2 carbon compounds by coupling to the triose phosphate dehydrogenase system (Racker, '48).

DPNase activity, splitting both the nicotinamide riboside linkage and the pyrophosphate linkage, was assayed according to Colowick, et al. ('51). Overall DPN destruction was estimated by the alcohol dehydrogenase method at 340 mμ. The decrease in cyanide addition to DPN at 325 mμ is a measure of the enzyme splitting the nicotinamide riboside linkage. The difference between the 340 mμ reading and that at 325 mμ represents pyrophosphate splitting activity. Cytochrome oxidase was determined according to the chlorophenol dye method of Smith and Stotz ('49). Ribonuclease and desoxyribonuclease was detected by change in optical density in the Beckman at 260 mμ following incubation at 37° C. for one hour.

ATPase was determined by the incubation of ATP with the brei. The reaction was stopped by placing in boiling water for one minute. The remaining ATP was then measured in the firefly luminiscent system (McElroy, '51).

## RESULTS

### *Glycolysis (Embden-Meyerhof scheme)*

Table 1 lists evidence for the glycolytic pathway in varieties 4 and 8 of *Paramecium aurelia* with the demonstration of phosphohexoisomerase, aldolase and triose phosphate dehydrogenase activites in both varieties. Preliminary findings indicate weak lactic dehydrogenase activity in variety 4. Variety 8 has not yet been tested for the latter.

As yet no hexokinase activity has been found in either variety, although the test system is reactive on the addition of crystalline hexokinase (Pabst Laboratories). This apparent lack of hexokinase may be only procedural as Simonsen and Van Wagtendonk ('52) have reported glucose stimulation of respiration in stock 51. Alcohol dehydrogenase activity is also lacking in both varieties and a cursory attempt to find glycerophosphate dehydrogenase in stock 51 proved negative.

Geddes and Humphrey ('51) reported the occurrence of glycolysis in *Paramecium caudatum* with the production of lactic but not pyruvic acid.

TABLE 1

*Glycolytic enzymes*

The activity of phosphohexoisomerase is given as micromoles of fructose-6-phosphate disappearance per milligram protein per hour at 37°C.[1] The unit of activity for aldolase and triose phosphate dehydrogenase is the change in optical density at 340 mμ in log $I_o/I_1$ of 0.001 per milligram protein per minute.

| ENZYMES | STOCK 51, VARIETY 4 | STOCK 131, VARIETY 8 |
|---|---|---|
| Phosphohexoisomerase | 2.92 | 3.35 |
| Aldolase | 82 | 77 |
| Triose phosphate dehydrogenase | 442 | 175 |

[1] The reverse reaction, the conversion of glucose-6-phosphate to fructose-6-phosphate was also observed.

TABLE 2

*Citric acid cycle*

The unit of activity for aconitase and isocitric dehydrogenase is defined as the change in optical density at 340 mμ in log $I_o/I_1$ of 0.001 per milligram protein per minute.

| ENZYMES | STOCK 51, VARIETY 4 | STOCK 131, VARIETY 8 |
|---|---|---|
| Aconitase | 170 | 38 |
| Isocitric dehydrogenase | 496 | 262 |

## *Citric acid cycle (Krebs cycle)*

Evidence for the operation of the Krebs cycle resides in the demonstration of aconitase and isocitric dehydrogenase in both varieties (table 2). The *Paramecium* isocritic dehydrogenase specifically requires TPN as a coenzyme. No reduction of DPN such as reported in yeast (Kornberg and Pricer, '51) was found.

*Paramecium caudatum* is known to have succinic dehydrogenase (Humphrey and Humphrey, '48; Preheim and Krueger, '50) and fumarase (Endahl and Krueger, '54).

### *Hexose monophosphate shunt (oxidative pathway for glucose – 6 – phosphate utilization)*

Stocks 51 and 131 grown under the conditions outlined above appear to lack the oxidative pathway for glucose – 6 – phosphate conversion through 6 – phosphogluconate to ribose – and ribulose – 5 – phosphate, the subsequent breakdown of the 5 carbon esters to triose phosphate, and its incorporation into the Embdem-Meyerhof scheme at the triose-phosphate dehydrogenase level. Extensive efforts to demonstrate the first two steps in this pathway resulted in failure. Similarly, no evidence for the formation of triose phosphate from the phosphorylated five carbon esters was obtained by coupling to the triose phosphate dehydrogenase system. Lastly, desoxyribose – 5 – phosphate formation from triose phosphate and acetaldehyde could not be found.

### *Miscellaneous enzymes*

We have confirmed Simonsen and Van Wagtendonk's report ('52) of cytochrome oxidase activity in stock 51 and extended Van Wagtendonk and Vloedman's ('51) evidence for ATPase in stock 51 by demonstrating its presence in both stock 51 and stock 131. Preheim and Krueger ('50) have shown cytochrome oxidase activity in *P. caudatum.*

Using our breis Wosilait, Nason and Terrell ('54) [4] found pyridine nucleotide-quinone reductase and pyridine nucleotide – 2 methyl, 1–4 naphthoquinone reductase (Wosilait and Nason, '54) in stock 51.

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[4] The species of *Paramecium* was mistakenly reported as *P. caudatum* in this paper. It should read *P. aurelia.*

In view of the destructive action of the cytoplasms of these varieties on foreign nuclei, those enzymes which effect nucleic acids and nucleotides are of particular interest. Evidence for the presence of desoxyribonuclease, ribonuclease, 2, 3, and 5 nucleotidases, and DPNase activity is given in table 3.

TABLE 3

*Enzymes acting on nucleic acids and nucleotides*

Desoxyribonuclease and ribonuclease activity is given as the change in optical density at 260 mμ in log $Io/I_1$ of 0.001 per milligram protein per hour. Nucleotidase activities are presented in terms of milligrams of inorganic phosphate released per milligram protein per half hour. See methods section for the determination of DPNase activities. These activities are given as the changes in optical density in log $Io/I_1$ of 0.001 per milligram protein per hour.

| ENZYMES | STOCK 51, VARIETY 4 | STOCK 131, VARIETY 8 |
|---|---|---|
| Desoxyribonuclease | $1.02 \times 10^4$ | $1.21 \times 10^4$ |
| Ribonuclease | $1.08 \times 10^4$ | $1.21 \times 10^4$ |
| DPNase | | |
| a. nicotinamide riboside linkage | 238 | 377 |
| b. pyrophosphate linkage | 104 | 287 |
| Nucleotidase | | |
| a. 2 adenylic | | |
| pH 7.6 | +[1] | 1.315 |
| pH 4.9 | + | 1.304 |
| b. 3 adenylic | | |
| pH 7.6 | + | .810 |
| pH 4.9 | + | .957 |
| c. 5 adenylic | | |
| pH 7.6 | + | 1.00 |
| pH 4.9 | + | 1.567 |

[1] These enzymes are known to occur in stock 51, but quantitative data are not available.

The activity of the enzyme attacking the nicotinamide riboside linkage of DPN seems greater than that of the pyrophosphate splitting enzyme in both varieties. In other respects the *Paramecium* DPNase is much like that found in *Neurospora.* It is inhibited by nicotinamide at high concentration but not by isonicotinic acid hydrazide (Zatman, et al., '52). Neither was any evidence found for the formation of the isonicotinic acid hydrazide analog of DPN (Zatman, et al., '53).

Table 3 also presents evidence for adenylic 2, 3, and 5 nucleotidase activities under both acidic and basic conditions in stock 31. Although quantitative data cannot be given, similar activities were observed for stock 51. It is not known whether these activities are due to more than one enzyme of a specific nature or to a non-specific phosphatase. These activities are largely inhibited at 37° C.

### *Mixing experiments*

Although the enzymology of the two stocks appears similar, it is conceivable that the protoplasm of each might contain material antagonistic to the normal functioning of some of the enzymes of the other stock, which in turn might cause the observed hybrid mortality. To test this possibility, breis of the two varieties were mixed and their activities compared with that of the individual breis. It is fully appreciated that breis may not accurately simulate the enzyme activities of whole cells.

The aldolase, triose phosphate dehydrogenase, isocitric dehydrogenase, ribonuclease, desoxyribonuclease, DPNase and nucleotidase activities of mixed breis were examined and no significant evidence of inhibition or stimulation was found. Indeed, the systems involved in respiration may not be intimately tied up with the cause of hybrid mortality as oxygen uptake of mixed breis in the Warburg respirometer is neither stimulated nor inhibited.

## DISCUSSION

Although varieties 4 and 8 are closely related in the evolutionary sense, the two stocks examined in this paper are known to manifest numerous genetic differences. Despite these genetic differences no qualitative enzyme difference between varieties have yet been found. Carbohydrate metabolism of both varieties is similar. Both are able to utilize glucose via the glycolytic system and citric acid cycle, but not through the oxidative pathway. As in *P. caudatum* lactic acid is probably the end product of glycolysis and not alcohol.

The apparent absence of the oxidative pathway for glucose – 6 – phosphate utilization leaves the whole problem of pentose sugar metabolism in *Paramecium* an open question. Perhaps the pentoses are available from the rather opulent diet the bacteria provide. Under more austere circumstances these protozoa may be able to form the pentoses for themselves.

The mixing experiments would seem to rule out a simple inhibition or abnormal activation as the cause of hybrid mortality. Death is more likely the result of a sequence of reactions. The metabolism of intervarietal conjugants which are doomed to die should prove very promising material in this connection.

The sublethal temperature for paramecia, in general, is around 37° C. As has been mentioned above, the adenylic nucleotidases are active at the more nearly optimum (for paramecia) temperature of 30° C., but almost entirely inactive at 37° C. One can hardly keep from speculating that such enzyme inactivations may be the underlying cause of temperature sensitivity.

## SUMMARY

Stock 51, mating type VIII, variety 4 and stock 131, mating type XV, variety 8 have been compared enzymatically. Some 30 enzymes have been studied and some 20 have been found: no qualitative differences have been found. Both varieties have phosphohexoismerase, aldolase, triose phosphate dehydrogenase and probably lactic dehydrogenase among the enzymes of glycolysis. Hexokinase, alcohol dehydrogenase and glycerol phosphate dehydrogenase were not demonstrated. Of the Krebs cycle enzymes, aconitase and TPN specific isocitric dehydrogenase were found. No evidence for the oxidative pathway for glucose – 6 – phosphate utilization was

obtained. In addition, cytochrome oxidase, ATPase, pyridine nucleotide-quinone reductase, pyridine nucleotide – 2 – methyl, 1–4 naphthoquinone reductase, desoxyribonuclease, ribonuclease, 2, 3 and 5 nucleotidase and DPNase activities have been demonstrated.

The enzymatic activities of mixed breis indicates that a simple inhibition or stimulation is not the cause of the hybrid mortality between these varieties.

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# COMPARATIVE STUDIES ON THE METABOLISM OF THE CULTURE FORM AND BLOODSTREAM FORM OF TRYPANOSOMA GAMBIENSE

THEODOR VON BRAND, EUGENE C. WEINBACH
AND ELEANOR JOHNSON TOBIE
*U. S. Department of Health, Education, and Welfare, Public Health Service, National Institutes of Health, National Microbiological Institute,*[1] *Bethesda, Maryland*

Our knowledge of the metabolism of trypanosomes is based primarily on studies of either the blood stream or the culture form of various species, the latter corresponding invariably to the forms appearing in the invertebrate vectors. So far, a detailed comparison between the various stages within the same species is lacking, although isolated observations indicate significant differences.

Thus, it has been shown that the culture forms of *T. cruzi* consume relatively large amounts of glucose while no significant sugar consumption could be demonstrated with its bloodstream stage (von Brand, Tobie, Kissling and Arams, '49). It has also been reported that the respiration of the culture form of *T. gambiense,* in contrast to that of the bloodstream trypanosome, is somewhat sensitive to cyanide (von Brand and Johnson, '47), and indications for differences in reaction to sulfhydryl inhibitors exist (von Brand, Tobie and Mehlman, '50).

The development of a medium in which *T. gambiense* can be grown *in vitro* in large numbers (Tobie, von Brand and Mehlman, '50) made the present study possible. Its purpose was to investigate the possibility of qualitative and quantitative differences between the culture form (corresponding essentially to the proventricular form of the tsetse fly) and the trypanosome in the bloodstream.

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[1] Laboratory of Tropical Diseases.

## MATERIAL AND METHODS

*1. Parasites.* The culture form studied was the *T. gambiense* Cheick strain[2] maintained since 1950 in culture according to Tobie, von Brand and Mehlman ('50). For the isolation and characterization of metabolic end products, large numbers of parasites were grown in tubes (200 × 25 mm) on diphasic medium (15 ml base, 10 ml overlay). After about 10 to 12 days incubation at 24°C. the supernatant of numerous tubes was decanted and pooled.

In those experiments where quantitative relationships between various metabolites were established, the parasites were cultured in rubber-stoppered Erlenmeyer flasks of 50 ml capacity containing 15 to 20 ml wholly liquid medium (Tobie, von Brand and Mehlman, '50). Initial samples were taken from freshly inoculated flasks and noninoculated control flasks and similar final samples after one week's incubation at 24°C.

For the RQ determinations, parasites grown in test tubes on diphasic medium were concentrated by centrifugation and suspended in an appropriate amount of overlay of the same medium.

The bloodstream form used was the Wellcome TS strain, an old laboratory strain maintained for years in white rats. The parasites were studied in most instances in whole blood withdrawn from the heart, diluted with blood of fresh rats to give a parasite density of 50 to 100 million per milliliter, and with glucose content raised to about 300 mg %. Heparin served as anticoagulant.

Because the determination of the RQ of the parasites would have been difficult in whole blood, these experiments were done with flagellates suspended in serum Ringer's solution. The parasites were separated from the blood elements by one or two slow speed centrifugations (125 × G) followed by one high speed centrifugation (2400 × G) and dispersion in a mixture of rabbit serum and Ringer's solution (1:1) with a glucose content of about 300 mg %.

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[2] The strain was obtained in 1950 through the courtesy of Dr. D. Weinman.

2. *General analytical methods.* The respiratory studies were conducted according to the standard Warburg technique, using vessels of approximately 6 ml capacity at either 29°C. (culture form) or 37°C. (bloodstream form). The RQ was determined by Warburg's direct method with due consideration of the bound carbon dioxide.

Glucose was determined according to the method of Hagedorn and Jensen ('23); pyruvic acid according to Friedemann and Haugen ('43), some of the determinations being checked by the more specific modification of Slavic and Michalec ('49). Barker and Summerson's ('41) method was used for lactic acid, Bueding's ('49) procedure for the total volatile acids. Succinic acid was determined manometrically (Umbreit, Burris and Stauffer, '49), a fresh 5% rat liver homogenate serving as the source of succinoxidase. McConnell's ('52) method for the determination of ethyl alcohol was used in the early experiment; it was replaced by the more sensitive method of Henry et al. ('48) in the remaining experiments.

Further procedures used in the isolation and identification of end products are described in the following section.

## RESULTS

### *Identification of acid end products*

*Culture form.* Two samples were carried through the following procedure, the first consisting of about 500 ml, the second of about 1,000 ml overlay of culture tubes. They were deproteinized with tungstate. After filtration and further acidification with $H_2SO_4$, the filtrates were exhaustively extracted with ether in a Friedrich extraction apparatus. A small amount of water and some solid sodium bicarbonate were added, and the ether evaporated at room temperature under constant stirring with a glass rod. The aqueous residue was filtered, acidified with $H_2SO_4$ and steam distilled in an all-glass distilling apparatus.

*Volatile acids.* The distillate containing the volatile acids was accurately neutralized with NaOH (bromthymol blue) and concentrated to a small volume by distilling off most of the water. The remaining water was removed at room temperature *in vacuo* over $CaCl_2$. The residue showed a faintly but distinctly positive spot test with chromotropic acid, indicating the presence of small amounts of formic acid (Feigl, '47). The lanthanum test for acetic and/or propionic acid was strongly positive (Feigl, '47). A preliminary fractionation of part of the first sample on a silica gel column according to the procedure of Ramsey and Patterson ('45) indicated the presence of at least 4 acids with the solubility characteristics of $C_1$, $C_2$, $C_3$, and $C_4$ acids.

The remaining material of the two samples was sent to Dr. E. Bueding to whom we are indebted for the following information. The material was fractionated on the celite columns developed by Bueding and Yale ('51). His analyses gave the following results:

Sample no. 1: Total acidity 1.13 milliequivalents. Analysis of a small aliquot (0.062 milliequivalent) revealed that over 97% consisted of acetic acid.

Sample no. 2: Total acidity 3.02 milliequivalents. Same result as sample no. 1 on fractionation of 0.072 milliequivalent. Fractionation of 1.5 milliequivalents, followed by subfractionation on two columns (at pH 7.6 and 6.5) gave the following:

| | |
|---|---|
| $C_5$ | 0.7% |
| $C_4$ | 0.8% |
| $C_3$ | 0.2% |
| $C_2$ | 98.3% |
| Total | 100 % |

No formic acid could be detected, but since the recovery of this acid is not quantitative, it is possible that small amounts could have been lost. Doctor Bueding prepared the p-bromphenacyl derivative of the $C_2$ fraction. After several recrystallizations it had the melting point of the corresponding acetate derivative (83 to 84 C.), and there was no depression of the melting point after mixing the derivative of the sample with that of authentic acetic acid. Found: C, 46.6; H, 3.7.

Calculated: C, 46.7; H, 3.5.[3] Acetic acid was unquestionably the main constituent of the volatile acid fraction.

*Nonvolatile acids.* The residue remaining in the distilling flask after removing the volatile acids was transferred to a Kutscher-Steudel extraction apparatus and extracted exhaustively with ether. The ether was transferred to a separatory funnel and shaken vigorously three times with an equal volume of distilled water. The third aqueous extraction was only faintly acid to pH paper. The combined aqueous solutions were evaporated and yielded a semi-crystalline residue.

*Determination of succinic acid.* Approximately 80 mg of the amber-colored, semi-crystalline residue was dissolved in a minimum amount of hot water. The amber-colored solution was treated with 80 mg of Norite, heated gently for a few minutes and filtered, using essentially the micro techniques described by Schneider ('46). Upon chilling the colorless filtrate, white crystals separated. These were collected by centrifugation and removal of the supernatant solution by aspiration. The recrystallization was repeated, omitting the treatment with Norite, to yield 30 mg of pure crystalline material identical with authentic succinic acid (MP 190 C.[4] There was no depression in the melting point of a mixture of the sample and authentic succinic acid. Found: C, 40.88; H, 5.23. Calculated for $C_4H_6O_4$: C, 40.68; H, 5.12).

The dianilide was prepared by treating a portion of the recrystallized sample with $PCl_5$, followed by conversion of the diacylchloride to the dianilide. It melted at 229°C., either alone or when mixed with a specimen prepared from authentic succinic acid.

A second portion of the crude semi-crystalline residue was purified by preparing the copper salt of the acid; subsequent conversion to the free acid yielded a crystalline product that was identical with authentic succinic acid with respect to melting point and elemental analysis.

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[3] We are indebted to Mr. Robert J. Koegel for performing the elemental microanalyses.

[4] All melting points were determined with the Fischer-Johns apparatus and are uncorrected.

*Absence of other dicarboxylic acids.* Portions of the crude residue from the ether extraction were examined by appropriate spot tests of Feigl ('47) and found negative for the following acids: Oxalic, malic, and fumaric. Traces of citric acid, found in the early experiments, arose from the solid phase of the medium containing citrated blood. When the trypanosomes were grown on a citrate-free medium, no evidence of metabolic citric acid was found. Where possible, additional tests were performed. Thus, no insoluble Ca salt could be obtained from the crude residue (absence of oxalic acid), and no decrease in the U.V. absorption at 240 mμ was observed after chemical reduction (absence of fumaric acid).

*Bloodstream form.* The following procedure was used to ascertain whether the bloodstream form produces succinic acid. Forty milliliters of blood containing 100 million parasites per milliliter were incubated in 5 ml lots in large Warburg flasks at 37°C. for 50 minutes during which time they consumed about 50 mg glucose. At the end of the incubation period the blood was deproteinized with tungstate, centrifuged, the supernatant further acidified with $H_2SO_4$, and extracted with ether in a Friedrich extraction apparatus. The ether was evaporated yielding a brown oily residue. No dicarboxylic acids could be detected in the residue when the fluorescein spot test of Feigl ('47) was applied to this material. Thus, it is apparent that the bloodstream form of *T. gambiense* did not form any appreciable amounts of succinic acid or other dicarboxylic acids under the *in vitro* conditions used.

### *Quantitative relationships*

*Culture form.* In expressing the results of metabolic experiments with trypanosomes, it has been customary to base the figures on a unit number of parasites because no adequate means exist of determining their weight accurately in each experiment. A similar procedure was not feasible with the

TABLE 1

*Quantitative determinations of metabolic end products of the culture form of T. gambiense*

| EXPERIMENTS NUMBER | GLUCOSE CONSUMED | METABOLITES PRODUCED | | MOLAR RATIO (Glucose 1.0) | CARBON BALANCE, PER CENT (Glucose 100%) |
|---|---|---|---|---|---|
| | *μg/ml* | | *μg/ml* | | |
| 11 | 480 ± 50.6 | Lactic acid | 41 ± 14.2 | 0.17 | 8.5 |
| 9 | 470 ± 55.5 | Pyruvic acid | 57 ± 7.3 | 0.20 | 13.3 |
| 10 | 517 ± 56.2 | Succinic acid | 114 ± 22.2 | 0.33 | 22.4 |
| 7 | 524 ± 36.2 | Acetic acid [1] | 145 ± 13.0 | 0.83 | 27.8 |
| 12 | 656 ± 62.8 | Ethyl alcohol | 0 | | |

[1] All volatile acids have been expressed here as acetic acid, the small amounts of $C_1$ and $C_3$ to $C_5$ acids being insignificant from a quantitative standpoint.

TABLE 2

*Metabolism of the blood stream form of T. gambiense. (All figures per 100 million/hr.)*

| MEDIUM | EXPERIMENTS NUMBER | OXYGEN CONSUMED | GLUCOSE CONSUMED | METABOLITES PRODUCED | | RQ |
|---|---|---|---|---|---|---|
| | | *mm³* | *mg* | | *μg* | |
| Blood | 4 | 179 ± 15 | 1.49 ± 0.16 | Volatile acids | 0 | |
| Blood | 4 | 177 ± 12 | 1.69 ± 0.21 | Ethyl alcohol | 0 | |
| Serum | 6 | 133 ± 13 | Not determ. | Respiratory $CO_2$ | 12 ± 3 | 0.09 ± 0.02 |

culture form of *T. gambiense* when a culture was analyzed at the time of inoculation and after a week's incubation. At this time the peak of population had, in most instances, already been passed, and thus it would not have been possible to arrive at a reliable "average" population figure. We based most of our quantitative considerations (excluding the respiratory experiments) therefore on the amount of glucose consumed by the parasites during the time of incubation.

The figures summarized in table 1 show that succinic and acetic acids were the main acids excreted while relatively little lactic and pyruvic acid was produced. No definite indications of ethyl alcohol formation [5] were found. The medium always contained small amounts of substance reacting like ethanol, but the values obtained after incubation were never significantly increased. The method of Henry et al ('48) especially is very sensitive, and we feel confident that an alcohol production exceeding 1 μg per milliliter would have been detected. The carbon balance shows that the identified end products account for 72% of the carbon of the metabolized glucose.

The flagellates consumed oxygen at a rate of 18 $mm^3$/100 million/hr., and their RQ was 0.97 ± 0.06 (7 experiments, 29°C.); that is, an appreciable amount of true respiratory carbon dioxide was given off. Its source may have been the carbon of the glucose not accounted for by the identified organic end products, but this cannot be proven. It is impossible to correlate the carbon dioxide determined in short term Warburg experiments with the amounts of glucose disappearing in long lasting culture experiments. It must also be emphasized that an RQ of nearly 1.0 does not necessarily indicate complete oxidation of a fraction of the carbohydrate consumed. The RQ is notoriously unreliable for such deductions in organisms gaining part of their energy from incomplete oxidations. It is entirely possible that some unrecognized partially oxidized compound (or compounds) was excreted in addition to the identified ones. As mentioned previously, the production of significant amounts of ethanol, oxalic, fumaric, malic, or citric acids has been ruled out.

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[5] We are indebted to Dr. M. Agosin for collaborating with the alcohol determinations.

*Bloodstream form.* Previous work has shown that the bloodstream form of *T. gambiense* does not produce measurable amounts of lactic acid (von Brand, Regendanz and Weise, '32), but that it does produce large amounts of pyruvic acid corresponding almost quantitatively to the sugar consumed (von Brand, Tobie, Mehlman and Weinbach, '53). These determinations were not repeated during the course of the present work. We did test quantitatively whether the bloodstream flagellates produce volatile acids or ethyl alcohol. For these determinations 2 to 3 ml of heavily infected blood were incubated in Warburg flasks at 37°C. for 50 minutes and initial and final determinations were carried out. Despite pronounced oxygen and glucose consumption (table 2), no evidence was found for the production of either volatile acids or ethyl alcohol. The oxygen and glucose values are in close agreement with those found previously for the same species (von Brand, Tobie, Mehlman and Weinbach, '53).

The respiratory quotient was determined in serum (table 2). In common with the figures reported for other African species (*T. rhodesiense,* Christophers and Fulton, '38; *T. equiperdum,* Reiner, Smythe and Pedlow, '36; *T. hippicum,* Harvey, '49), it was found to be extremely low, averaging 0.09 (extremes 0.06 and 0.15). It follows that small amounts of true respiratory carbon dioxide were regularly produced which would not be the case if the glucose to pyruvate pathway were the only one functioning. The sources of this carbon dioxide have not been elucidated.

## DISCUSSION

The present study has shown profound quantitative and qualitative metabolic differences between the culture and the bloodstream form of *T. gambiense.* The former consumed at 29°C. 18 mm³ $O_2$/100 million/hr., the latter at 37°C. just 10 times as much. Since the temperature relationship of the culture form is quite normal, that is, since its $Q_{10}$ is in the vicinity of 2 (von Brand and Johnson, '47) such a difference cannot be due to the difference in environmental temperature.[6] A similar situation unquestionably exists in respect to the rate of glucose consumption. This cannot be proven quantitatively because the glucose consumption of the cultured flagellates, in contrast to that of the bloodstream trypanosomes, is too low to be determined accurately in short term experiments with known numbers.

The metabolic end products resulting from the glucose utilization are different in both cases. In the bloodstream form glucose is nearly quantitatively degraded to pyruvic acid. The culture form, on the contrary, produced little pyruvic acid. Large amounts of succinic and acetic acids, as well as small amounts of $C_1$, $C_3$, $C_4$, and $C_5$ volatile acids and lactic acid appeared instead. The metabolic end products of the culture stage are hence on the whole similar, though differing in details, from those reported for the culture form of other Trypanosomidae (*T. cruzi,* Chang, '48; *Leishmania donovani,* Crowther, Fulton and Joyner, '54). The RQ of the culture form was quite high in contrast to that of the bloodstream trypanosome which was extremely low. These differences in RQ probably are associated with the two different degradation levels of glucose utilization.

The culture form is metabolically more versatile than the blood stream form. The latter's metabolism, though from a formalistic standpoint simpler, is undoubtedly not an original condition (von Brand, '53), but it represents a secondarily acquired type. Whether a true loss of enzymes is involved or whether the "lost" enzymes are present in an inactive form, remains to be studied. It has been found that the bloodstream form of *T. hippicum* definitely lacks such enzymes as succinic or malic dehydrogenase (Harvey, '49). This does not necessarily prove that an identical situation prevails in

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[6] The $Q_{10}$ of the bloodstream form of *T. gambiense* has not been determined. In the closely related *T. rhodesiense* it is in the vicinity of 2 (von Brand and Johnson, '47).

*T. gambiense.* The former species, in contrast to the latter, is transmitted purely mechanically and has apparently lost the capability of developing in insects or cultures. Recent studies on changes in metabolism of *T. gambiense* and *T. rhodesiense* made resistant to arsenicals (von Brand, Tobie, Mehlman, and Weinbach, '53; Williamson, '53) seem to point towards a reserve of latent enzymes (or the rapid development of adaptive enzymes) in the bloodstream stage of these parasites.

The life cycle of *T. gambiense* is complicated. The present investigation has demonstrated metabolic differences between the bloodstream trypanosome and the culture form, the latter corresponding essentially to the forms developing in the gut of the tsetse fly. It is unfortunate that at present no medium exists which would allow the completion of the life cycle and thus permit a study of other stages. The most glaring gap in our knowledge of the physiology of this flagellate is lack of information as to the metabolic characteristics of the salivary gland forms.

## SUMMARY

1. The culture form of *T. gambiense* consumes much less oxygen and glucose than the bloodstream stage, even after allowances are made for the different environmental temperatures.

2. The RQ of the culture flagellates is high, nearing unity, while that of the bloodstream form is extremely low.

3. The metabolic end products of the culture flagellates are primarily succinic and acetic acids, while small amounts of pyruvic and lactic acids, as well as $C_1$, $C_3$, $C_4$, and $C_5$ volatile acids are also produced. These end products account for 72 percent of the carbon atoms of the metabolized glucose. The bloodstream trypanosomes, on the other hand, produce only pyruvic acid which accounts nearly quantitatively for the glucose consumed. They form no lactic, succinic, or volatile acids. Neither stage produces ethyl alcohol, citric, oxalic, malic, or fumaric acid.

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# INORGANIC POLYPHOSPHATASE IN THE DEVELOPING FROG [1]

GEORGE G. BERG [2]
*Department of Zoology, Columbia University, New York, N. Y.* [3]

ELEVEN FIGURES

Inorganic polyphosphatases have been found in vertebrate organs (Kitasato, '28 a,b; Neuberg and Fischer, '38; Barnabei et al., '50; Mattenheimer, '51; Malmgren, '52) but have no known function in vertebrate metabolism. The work reported here was, therefore, directed at finding clues to the physiological role of the enzymes. Two related questions were asked: one, can vertebrate inorganic polyphosphatases be traced back to early development by analytical means, and two, can they be stained histochemically? In tests done on the frog positive answers were found on both counts. The biochemical assay served to describe the relation between inorganic polyphosphatases and known organic phosphatases in development. The newly developed histochemical method permitted an anatomical inquiry into the function of the enzymes in the organism.

## I. Biochemical experiments

### INTRODUCTION

The various phosphate-splitting enzymes differ widely in their response to embryonic development. The content of neutral polyphosphatase does not change at all in the post-gas-

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[2] Submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy, in the Faculty of Pure Science, Columbia University.

[3] Present address: Atomic Energy Project, The University of Rochester, Rochester 20, New York.

trula sea urchin embryo, while alkaline apyrase increases slowly, and alkaline phosphatase increases exponentially (Gustafson and Hasselberg, '51). This sharp increase in alkaline phosphatase has been found in all tests of vertebrate and echinoderm post-gastrula embryos (Moog, '46; Mazia et al., '48; Gustafson and Hasselberg, '50; Krugelis, '50). An extremely rapid increase of alkaline phosphatase also takes place in the organogenesis of the duodenum in the chick and mouse, where a phosphatase-synthesizing mechanism is activated by hormonal control (Moog, '50, '51, 52a,b). The histochemical pattern of distribution of alkaline phosphatase suggests that enzyme accumulation in the post-gastrula amphibian embryo takes place primarily in the mesenchyme of organ primordia (Brachet, '46; Osawa, '51), while the later increase in the maturing duodenum is due to changes in the endodermal components (Moog, '50, '51).

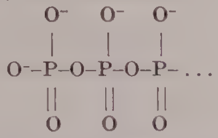
The post-gastrula embryo and developing duodenum of the frog were selected here as materials for testing the response of acid inorganic polyphosphatase to development. Parallel assays of alkaline glycerophosphatase were made throughout. To gain a direct measure of distribution where only histochemical estimates were available, the endodermal and mesectodermal components of embryos were analyzed separately. The data on embryonic yolk then served as the first entry in the developmental series consisting of the prefunctional duodenum, functional tadpole duodenum and adult duodenum.

The assay method was based on tests of pH optima of inorganic polyphosphatase which were made in the embryonic and adult frog, and on tests of the activation of the enzyme by magnesium.

## MATERIAL AND METHODS

1. *Substrate.* Inorganic sodium polyphosphate (IPP) purchased under the label "Sodium Metaphosphate" from Eimer and Amend was the substrate. Commercial "meta-

phosphate'' (Merck Index, '52) is a mixture of linear polymers of phosphoric acid (polyphosphates) of the form:



$$\mathrm{O^{-}{-}\underset{\underset{\displaystyle O}{\|}}{\overset{\overset{\displaystyle O^{-}}{|}}{P}}{-}O{-}\underset{\underset{\displaystyle O}{\|}}{\overset{\overset{\displaystyle O^{-}}{|}}{P}}{-}O{-}\underset{\underset{\displaystyle O}{\|}}{\overset{\overset{\displaystyle O^{-}}{|}}{P}}{-}\ldots}$$

and contains no ring or ''meta'' compounds (Ebel, '52a; Thilo and Rätz, '49). Estimates of the molecular size of the polymers range from n = 13 (Ebel, '52a) up, reaching over four hundred times that size (Tria, '51). The biochemical substrate solutions used in assaying the enzymatic activity of homogenates contain not only the polymers dissolved in them initially, but also the breakdown products, down to n = 1 (Ebel, '52a). Barnabei et al. ('50) estimated the rate of breakdown of IPP in solution to phosphate at 1% in 48 hours.

In this laboratory IPP was kept in stock solutions ranging from 0.1% to 3.3%. These solutions were neutral or mildly acid (pH 5.5–7.5) and contained free (ortho) phosphate. Within the first two weeks of storage at 4° C. the free phosphate rose from an initial 5 or 6% of the total phosphate to 10 ± 2% of the total; it then remained at the same level for as long as two months.

2. *Procedure.* *Rana pipiens* adults, laboratory-reared embryos and larvae were used. Adult organ samples or whole embryos were ground with distilled water in a Potter homogenizer on ice. Embryonic parts were pooled in a micro-homogenizer on ice and ground with distilled water. A mixture consisting of equal parts of homogenate, buffer and substrate was incubated for one hour at 37.2° C.; the reaction was stopped with two parts of a solution of 0.4% uranium acetate in 10% trichloracetic acid (Sze, unpublished). After centrifugation, the total supernatant was removed for assay with a fine-tipped capillary pipette.

The buffers used are listed in table 1; they maintained the incubating mixture within 0.1 unit of their rated pH value.

TABLE 1

*Buffers for assay of phosphatase in homogenates*

| EXPERIMENTAL RANGE: pH | BUFFERING SUBSTANCE AND ITS CONCENTRATION | pK | COMPOSITION OF BUFFER | |
|---|---|---|---|---|
| | | | Solution I | Solution II to titrate |
| 1.4–3.4 | Glycine, 0.3 M | 2.35 | Glycine, 0.3 M in 0.3 M NaCl solution | HCl, 3 M |
| 3.8–5.7 | Acetate, 0.3 M | 4.73 | Acetic acid, 0.3 M | Na acetate, 0.3 M |
| 5.2–7.0 | Histidine, 0.3 M | 6.10 | Histidine KCl $H_2O$, 0.3 M | Histidine HCl $H_2O$, 0.3 M |
| 7.2–7.8 | Histidine, 0.15 M in half saturated barbital | | Histidine KCl $H_2O$, 0.3 M mixed with equal volume of Na barbital buffer, pH 7.9 | HCl, 3 M |
| 7.9–8.9 | Barbital, saturated | 7.91 | 6% Na barbital | 0.3 N HCl, and filter |
| 8.9–12 | Glycine, 0.3 M | 9.78 | Glycine 0.3 M in 0.3 M NaCl | KOH 3 M |

Three kinds of controls were used. Those in the first group were incubated without substrate, but substrate was added immediately after the stopping solution. Those in the second group had a heat-inactivated homogenate. In the third group the stopping solution was added prior to incubation. Groups 1 and 3 were used to estimate the phosphate released enzymatically from endogenous substrates of the tissue. In all controls, when one of the components of the incubating mixture was omitted, water or saline was added in its place. The total supernatant was recovered for assay, as in the experimental tubes.

The assay of phosphate followed the method of Berenblum and Chain ('38) as modified in this laboratory by Sze (personal communication). The modified method used microinstrumentation (Barth and Sze, '51); the colored isobutyl alcohol was not diluted with ethyl alcohol, but was cleared of water droplets by centrifuging directly in the colorimetric cuvette. This eliminated the need for a quantitative transfer of isobutyl alcohol to the colorimetric cuvette and for a quantitative dilution with ethyl alcohol in the cuvette, two potential sources of error. Readings were made on a Coleman Junior spectrophotometer at 700 mμ in a range calibrated for a linear relation between phosphate content and optical density.

A micro-assay and a macro-assay were used. In the micromethod, buffer, homogenate and substrate were placed in the incubation vessel in 10 λ (microliter) aliquots. The color was developed in the reaction vessel in either 0.5 cc or 1.0 cc of isobutyl alcohol. When 0.5 cc were used, the readings were linear up to 1.5 γ (microgram) P (capacity of the method) and the accuracy of readings on the spectrophotometer was ± 0.01 γ P. When 1.0 cc were used the capacity was 3γP and the readings accurate to ± 0.02γP. In the macro-method three 0.2 cc aliquots were used and the color developed in 3 cc of isobutyl alcohol. The capacity of the method was 6γP, and the readings accurate to ± 0.03γP.

The 10% level of initial free phosphate, found in the substrate solution, set a maximum to the concentration of substrate which could be used efficiently in the incubation mixture, since it caused high control readings. In the 10 λ aliquots of the micro-method 0.33% IPP was the strongest concentration used; the free 10% of the total phosphate amounted to 1 γP while the capacity of the method was 3 γ. Similarly, in the 200 lambda aliquots of the macro-method 0.03% IPP was used; it contained (at the 10% average) 1.8γ orthophosphate P, amounting again to almost a third of the total capacity of the method.

The glycerophosphate (GP) substrate was made up to the same total phosphate concentration as the IPP substrate; the concentration was 1% of sodium glycerophosphate · $5H_2O$ (Eastman) in the 10 λ aliquots of the micro-method. A pH of 9.4 was used for assay of alkaline GP-ase activity (Ponz, '46). All GP-ase substrates contained 0.012 M magnesium (chloride or sulfate) as activator, giving the final concentration of 0.004 M Mg in the incubating mixture (Kind and Macchi, '52).

Enzyme activity was computed per unit dry weight of tissue. In the micro-method, the 10 λ samples of homogenate for drying and weighing were placed on aluminum foil pans (Sze, '53) with the pipette which was used for charging the incubation vessels; pans were dried overnight at 120°C. A quartz spring microbalance was used; the quartz helix had a travel of 16 cm, corresponding to a capacity of 17.8 mg. The pans weighed from $\frac{1}{4}$ to $\frac{1}{2}$ mg, and the balance was calibrated for pan loads up to 240 γ without deviating from linear response (Sze, '53). Displacement was read with a cathetometer accurate to 0.02 mm, corresponding to 0.222 γ, and readings were reproducible to better than 1 γ. The balance was sensitive to changes in air density. This flotation effect was compensated for, before each series of weighings, by taking a zero reading with a set of tares similar to the weighing pans.

## RESULTS

*1. Activation of inorganic polyphosphatase* —

IPP-ase was activated by 0.004 M $Mg^{++}$ (table 2). This activator, in the form of $MgCl_2$ was added to all subsequent experiments.

*2. pH optimum of inorganic polyphosphatase* —

*(a) Acid and neutral IPP-ase in the adult duodenum.* The pH-activity curve of the duodenal IPP-ase showed two peaks, one at pH 4.2–4.6, the other at pH 7.2–7.6 (fig. 1). In the absence of experimentally added IPP substrate no significant amount of phosphate was released. Five series of determinations were made to cover the range from pH 1.9 to pH 9.5; each at from three to five pH values. For each determination a segment of the duodenum of one animal was used. Each preparation differed from the others in enzyme content per unit dry weight, although in each animal the shape of the pH curve was the same. Therefore the curve was plotted on a percentage basis, using the activity of each preparation at pH 4.4 as 100 (fig. 1).

TABLE 2

*Magnesium activation of inorganic polyphosphatase in adult duodenum*

Each entry represents the average of two determinations and their average deviation from the mean. Activity is measured in micrograms of phosphorus liberated per milligram dry weight of tissue in one hour. Tissue concentration in the incubation mixture is 0.55 mg dry weight per cubic centimeter; substrate concentration is 0.1 mg of sodium polyphosphate per cubic centimeter.

| pH | ACTIVITY: without Mg | $P \cdot mg^{-1} \cdot h^{-1}$ with 0.004 M Mg |
|---|---|---|
| 3.9 | 2 (± .6) | 16 (± 3) |
| 7.5 | 2 (± .2) | 26 (± 3) |

*(b) pH optimum in embryos.* Whole embryos, Shumway stage 18 (muscular response), were used. Two peaks of IPP-ase activity were found: one at pH 3.4–4.4, the second at pH 6.7–7.7 (fig. 2). In the absence of experimentally added substrate, a large amount of phosphate was released endogenously by the homogenates. This endogenous activity,

known to be due to phosphoprotein phosphatase in the acid range (Harris, '46), had an acid peak at pH 4.9–5.9 and an alkaline peak at pH 8.9–9.9 (fig. 2).

The determination over the entire range was made on eggs from a single clutch and plotted on the basis of absolute enzyme activity.

*3. Inorganic polyphosphatase and glycerophosphatase in adult duodenum* —

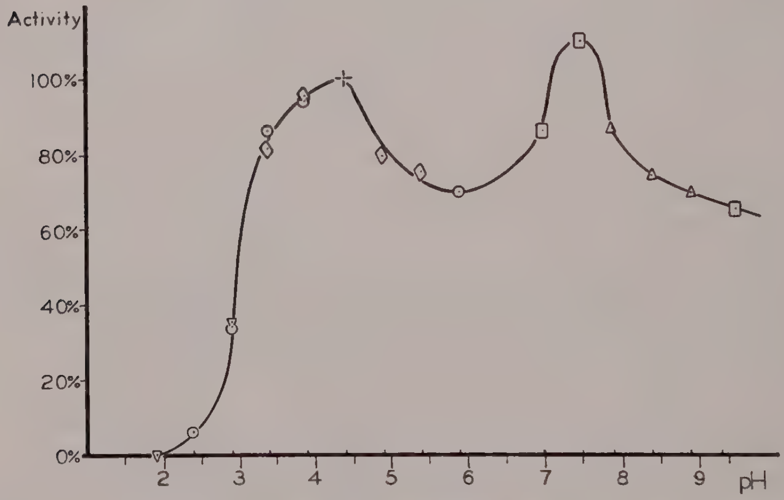


Fig. 1 pH curve of inorganic polyphosphatase activity in adult duodenum. Abscissa: enzyme activity as percent of the activity of the same preparation at pH 4.4. Ordinate: pH. The five symbols represent five preparations, each consisting of a homogenized duodenum of one frog. Each point represents the average activity of two samples of the same homogenate.

Individual variation in IPP-ase activity in adult duodenum was accompanied by a similar change in alkaline GP-ase activity. GP-ase was assayed in the same way as IPP-ase; total phosphate concentration was the same (0.01 M in the micro-method) for both substrates. In three experiments GP-ase at pH 9.4 was from 1.4 to 1.6 times more active than IPP-ase at pH 4.4. In three more experiments GP-ase at pH 9.4 was from 2 to 2.3 times more active than IPP-ase at pH 9.4. When IPP-ase activity at pH 9.4 was converted to the value at pH 4.4 (by using the ratio of 0.6 found in fig. 1), the ratio of GP-ase to IPP-ase remained in the 1.2 to 1.6 range in all 6 specimens, although the activity of each enzyme varied by a factor of 4 (GP-ase activity ranged from 7.2 to 29 γ P per milligram dry weight of tissue per hour).

There was no GP-ase activity at pH 3.9.

4. *Enzyme activities in embryonic tissues and in organogenesis* —

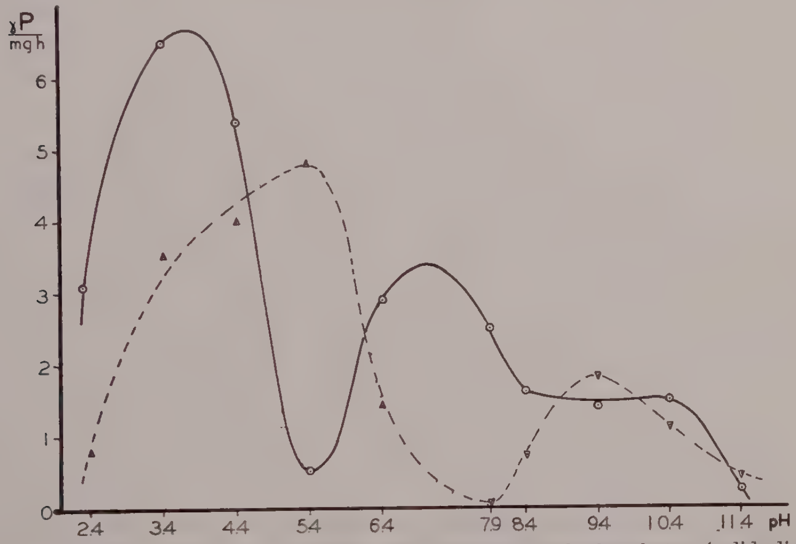


Fig. 2 pH curves of hydrolysis of inorganic polyphosphate (solid line) and of endogenous phosphorylated substrates (dotted line) by homogenates of Stage 18 (muscular movements) embryos. Abscissa: micrograms P. liberated per mg. dry weight per hour. Ordinate: pH. All determination are made on embryos of one clutch. Each point represents the average of from two to six readings.

(*a*) *IPP-ase and GP-ase in embryonic development.* Embryos ranging from stage 15 (neural crest) to stage 19 (heart beat) were used. The trunk was separated from the head and tail and dissected into two fractions: the yolk mass and the mesectoderm. Each fraction was pooled from dissections of 10 to 25 embryos of a single clutch. IPP-ase (pH 3.4), GP-ase (pH 9.5), acid GP-ase and phosphoprotein phosphatase were tested.

Each of the determinations shown in table 3 was done on a different clutch of eggs; the variability in enzyme content between clutches tended to obscure the changes due to development. There was, however, clearly more IPP-ase than GP-ase activity in both fractions of the embryo at stage 15 (rotation) and 16 (neural tube), and more GP-ase than IPP-ase at stage 19 (heart beat) in the mesectoderm only.

In the yolk fraction, IPP-ase activity remained fairly constant with age until stage 19; throughout it was higher than the GP-ase activity. In the mesectodermal fraction the IPP-ase activity increased with age, but the GP-ase activity increased faster, so that after stage 17 there was more GP-ase than IPP-ase activity (table 3).

TABLE 3

*Distribution of inorganic polyphosphatase and glycerophosphatase in embryos*

Each reading is an average of determinations on two samples of a single homogenate. Readings in each column represent one group of embryos. Activity is measured in micrograms of phosphate liberated per milligram dry weight of tissue in one hour $(P \cdot mg^{-1} \cdot h^{-1})$.

| Stage | | | | | |
|---|---|---|---|---|---|
| Shumway number | 15–16 | | 17 | 19 | |
| Equivalent hours at 18°C. | 67–72 | | 84 | 118 | |
| Number of animals | 19 | 15 | 10 | 25 | 12 |
| Yolk fraction, activity | | | | | |
| IPP-ase, pH 3.4 | 2.9 | 3.0 | | 1.2 | 3.0 |
| GP-ase, pH 9.4 | 0.3 | 0.1 | | 1.1 | 1.6 |
| Mesectoderm fraction, activity | | | | | |
| IPP-ase, pH 3.4 | 3.5 | 2.3 | | 6.5 | 5.6 |
| GP-ase, pH 9.4 | 1.4 | 0.6 | 7.1 | 9.6 | 11.0 |

(b) *Other phosphatases in embryonic material.* Acid glycerophosphatase (pH 5.4) was assayed in three of the embryonic fractions used above. It was detected only in the mesectodermal fraction of stage 19; its activity was 0.9 γ P/mg · hour as compared to the activity of 11.0 γ P/mg · hour of alkaline phosphatase in the same fraction (table 3). In the yolk fraction of the same clutch, and in the mesectodermal fraction of stage 16 (table 3), acid phosphatase activity was undetectable (the test was sensitive to 0.2 γ P/mg · hour and to 0.1 γ P/mg · hour respectively).

Acid phosphoprotein phosphatase (PPP-ase) in the same three fractions at pH 5.4 remained at the 3.2–3.6 γ P/mg · hour level of activity. At the same optimal pH in two determinations on whole stage 18 embryos, PPP-ase was 4.6 and and 5.1 γ P/mg · hour (average 4.8, fig. 2). Acid PPP-ase activity tested at pH 3.4 (below its pH optimum) in the mesectodermal fraction of stages 15, 16 and 19 ranged from 2.5 to 2.9 γ P/mg · hour.

Alkaline hydrolysis of endogenous substrate (shown in fig. 2) was tested at pH 9.4 in the embryonic fractions listed in table 3. Activity was found in all fractions; there was no significant difference between the enzymatic activities of the two embryonic regions, or between activities of embryos of one clutch tested at two different stages (stages 15 and 19). Activity varied widely from clutch to clutch (range: 0.1 to 2.3 γ P/mg · hour).

*(c) IPP-ase and GP-ase in the organogenesis of the duodenum.* Activity of acid IPP-ase and alkaline GP-ase was assayed in the thick-walled, yolky duodenum of the stage 24 (operculum closing) tadpole, and in the yolk-free duodenum of the stage 25 (operculum complete) tadpole. Embryos of the same clutch were used for both determinations. The findings were collated with enzyme activities in embryonic yolk and in adult duodenum (table 4). For this purpose, the data of the 4 experiments on embryonic yolk (table 3) were pooled, and standard deviation computed. The same was done with three experiments on adult duodenum in which both IPP-ase at pH 3.4 and GP-ase at pH 9.4 were assayed.

The activity of IPP-ase of the yolky duodenum was significantly higher than that of the yolk and significantly lower than that of the adult. Activity of the functioning tadpole duodenum was the same as that of the adult organ.

GP-ase activity of the yolky duodenum was low, but within the range of activity of embryonic yolk. GP-ase of the functioning tadpole duodenum, on the other hand, was extremely high, well outside the range of adult duodenum, as well as that of yolk.

Thus, the level of IPP-ase activity increased gradually in the duodenum from the embryonic primordium, through the differentiating non-functional organ, and became constant at the end of differentiation. GP-ase activity remained low in yolk and in the yolky non-functional organ; it reached an extremely high level at the beginning of function; and it was maintained at a high level in the adult.

TABLE 4

*Inorganic polyphosphatase in the organogenesis of the duodenum*

Each experiment was done on one clutch or one adult. Data for stages 15 to 19 are computed from table 3. Data for stages 24 (12 animals) and 25 (14 animals) are on members of one clutch. Activity is measured in micrograms of phosphate liberated per milligram dry weight of tissue in one hour ($P \cdot mg^{-1} \cdot h^{-1}$).

| ORGAN | STAGE (Shumway) | NUMBER OF EXPERIMENTS | IPP-ase activity pH 3.4 Average ± S.D. | GP-ase activity pH 9.4 Average ± S.D. |
|---|---|---|---|---|
| Yolk | 15–19 | 4 | 2.5 ± 1.1 | 0.8 ± 0.7 |
| Yolky duodenum | 24 | 1 | 5.9 . . | 0.2 . . |
| Herbivorous duodenum | 25 | 1 | 11.4 | 66.0 . |
| Adult duodenum | adult | 3 | 13.6 ± 2.5 | 25.7 ± 4.1 |

## II. Histochemical experiments

### INTRODUCTION

To show that an enzyme may be used for a physiological function, it is necessary to find it in a structure specialized for this function. The precision with which this can be done is as great as the accuracy with which a histochemical stain can be shown to reflect the actual location of the enzyme in the living cell. However, to postulate that the enzyme is not only utilizable but is also required, it is necessary to show that it is not absent from any of the sites at which the associated metabolic process takes place in the organism. Where, as in the case of vertebrate inorganic polyphosphatase, there is no basis for restricting the study to any one phase of physiology, it becomes necessary to find all the sites of low, as well as high, enzyme activity in the entire organism.

The tadpole in the final premetamorphic stage was selected for the study of acid IPP-ase. It had the advantages of small size and sectionable skeleton; and it contained all major tissue components of the adult with the exception of the genital system. The presence of the water-respiratory system proved to be of special advantage; and the yolk which caused staining artifacts was absent.

TABLE 5

*Comparative distribution of acid inorganic polyphosphatase and alkaline glycerophosphatase in representative adult organs*

(+) and (—) denote presence and absence of stain

| ORGAN | PATTERN OF IPP-ase | PATTERN OF ALKALINE GP-ase |
|---|---|---|
| Mesonephros | (+) cortex (proximal tubules)<br>(—) medulla (glomerulus) | (+) cortex (proximal tubules)<br>(—) medulla (glomerulus) |
| Duodenum | (+) columnar epithelium | (+) columnar epithelium |
| Tongue | (+) epithelium and glands | (+) epithelium and glands |
| Stomach | (+) epithelium and glands | (—) all tissues |
| Heart | (—) all tissues | (+) ventricle endothelium |

Acid IPP-ase was also stained in some adult organs. The study of the adult duodenum complemented the biochemical data on organogenesis.

Alkaline glycerophosphatase was tested in alternate sections of the same adult organs and tadpoles. It was also examined in histological preparations of the embryonic material used in Part I; these stains served to localize the sources of biochemically measured activity.

## MATERIAL AND METHODS

Specimens of tissue from *Rana clamitans* adults and *Rana pipiens* adults, and whole tadpoles in stage 25 were examined. Alcohol-acetone fixation and freeze-substitution fixation were used for acid IPP-ase; only alcohol-acetone fixation for alkaline GP-ase. Tissues were preserved and substrates prepared as described by Berg ('55). Descriptions of enzyme distribution were based on the pattern found after the shortest incubation time which gave a strong stain. No statements of enzyme localization were made where a slow and weak stain was adjacent to a strong stain, or where the stained structure has been shown to have an affinity for artifact staining.

## RESULTS

*1. Acid IPP-ase and alkaline GP-ase in the adult* —
Examples of adult organs showing the same pattern of distribution of the two enzymes, and of adult organs containing one enzyme and not the other are shown in table 5. The mesonephros and duodenum had the highest concentrations of both enzymes in the same cells, but the enzymes differed in intracellular distribution. In the mesonephros the epithelial cells of the proximal convoluted tubules contained both enzymes. The transition from the positive portion of the tubule to the negative portion (more distal from the glomerulus) occurred at the same site for both enzymes (in addition, however, the vascular bundles of the glomerulus had a weaker GP-ase reaction and no IPP-ase). GP-ase was concentrated in the brush border of tubule epithelium. IPP-ase was absent from the brush border but abundant in the cytoplasm. In the duodenum, the columnar mucosa contained acid IPP-ase and alkaline GP-ase. GP-ase was localized in the cuticular border. IPP-ase in the same cells was localized in the cytoplasm. Neither enzyme was found in the mucus of the goblet cells. Photographs of preparations of mesonephros and duodenum are shown in a prior publication (Berg, '55).

The tongue had a parallel distribution of the two enzymes. They were present in the epithelium covering the entire surface of the organ including the glands on the ventral side.

The stomach contained acid IPP-ase and no alkaline GP-ase. The enzyme was present in the glands and the surface epithelium.

*2. Distribution in the stage 25 tadpole* —

In the tadpole (stage 25 of Shumway) acid IPP-ase was confined to the cells of three organ systems: water respiratory, digestive, and excretory. IPP-ase was present in the linings of nares, pharynx, gills, opercular cavity and lumen of the entire digestive tract. It was also present in the pancreas and liver and lined the proximal part of the pronephric tubule. Over this entire area only some gill and pharyngeal elements, the small intestine, pancreas, liver and pronephros were stained by both acid IPP-ase and alkaline GP-ase. The distribution of IPP-ase and its relation to GP-ase are summarized in table 6.

TABLE 6

*Distribution of acid IPP-ase in the tadpole (stage 25) and its relation to alkaline GP-ase*

(+) and (—) denote presence and absence of GP-ase stain

| ORGAN SHOWING POSITIVE IPP-ase STAIN | GP-ase STAIN IN SAME ORGAN | | FIGURES |
|---|---|---|---|
| | In same cells | In other cells | |
| Nasal capsule | — | + | |
| Buccal cavity | — | — | |
| Gills | — | + | 3, 4 |
| Lining of pharynx | — | — | 3, 4 |
| Opercular chamber | — | — | 3, 4 |
| Pronephros | + | — | 5, 7 |
| Oesophagus | — | — | 5, 11 |
| Stomach | — | — | 6, 10, 11 |
| Small intestine | + | — | 5, 6, 8<br>9, 11 |
| Large intestine | — | — | |
| Pancreas | +/— | + | 6 |
| Liver | — | + | 5, 11 |

(*a*) *Water respiratory system.* In the nasal capsule, IPP-ase was present in the sustentacular (columnar) epithelium of the sensory lining. GP-ase was absent from the capsule proper, and present only in the basal cells. The squamous and ciliated epithelia of the pharynx, gill slits and epiglottis and the squamous epithelium of the opercular cavity were all rich in IPP-ase (fig. 3) which appeared to fill the cytoplasm (but not the cilia). GP-ase was absent. In the gills IPP-ase was present in the entire squamous epithelial covering, while GP-ase could be found only in the vascular bed and the perichondrium of the skeletal elements (fig. 4). The only overlapping of the two enzymes occurred at the extreme tip of the gills and in a few spots in the pharynx, where there may have been some GP-ase in the epithelium.

*(b) Excretory system.* In the pronephros, as in the mesonephros of the adult, both IPP-ase and GP-ase were present in the same cells of the nephron. The negative portion of the pronephric tubule could be traced proximad from the Wolffian duct and collecting tubule to the distal elements of the convoluted portion (fig. 5). The positive portion of the tubule could be traced distad from each of the three strongly staining nephrostomes through the proximal convoluted elements to a point of sharp termination of the stain. IPP-ase filled much of the cytoplasm of the tubular epithelium, beginning at the base of the cell, and was absent from the brush border (figs. 5, 7). GP-ase filled the brush border and was present in the cytoplasm that underlies it.

*(c) Digestive system.* In the ciliated epithelium of the oesophagus (figs. 5, 6) the epithelia of the stomach (fig. 6) and small intestine (figs. 5, 8), and the epithelium of the large intestine, the distribution of IPP-ase followed the pattern described for the adult duodenum. The enzyme filled a band of cytoplasm under the cell membrane at the lumen, and was absent from the cell membrane proper and all associated structures (cuticle, fig. 8; cilia). Occasionally, IPP-ase was found in the entire cytoplasm of an epithelial cell. In the stomach, in addition to the pattern in the surface epithelium, the stain was found in the cells of the tubules of chief glands, and in a few similar cells in the neck of the glands; it filled the cytoplasm in both cases (figs. 7, 10). GP-ase of the digestive tract was concentrated in the cuticu-

lar border of the epithelium and limited to the small intestine (figs. 9, 11).

There was relatively less IPP-ase and more GP-ase in the liver than in the pancreas. In the liver (fig. 5), IPP-ase was found in the sinusoid-lining cells which appeared completely filled with enzyme, and in the cuboidal epithelial lining of tubules where it formed a band in the part of cytoplasm most distal from the lumen. GP-ase, on the other hand, was most concentrated in the liver canaliculi and in the lining of the gall bladder and bile ducts; it was also present in the hepatic cord cells. In the pancreas (fig. 6), IPP-ase appeared in large cells uniformly scattered through the organ. GP-ase was strongest in the exocrine tubules and had only a weak reaction in all the gland cells.

*(d) Other sites of alkaline glycerophosphatase activity.* In the absence of a prior description of GP-ase distribution in the tadpole, a note may be made of its unusual distribution in some additional tissues. There was a reverse axial gradient in the central nervous system, with a high enzyme level in the caudal portion and some strongly staining specialized areas in the brain (fig. 4). The striated musculature was rich in GP-ase although, as is known, it is very poor in GP-ase in the adult (Kind and Macchi, '52).

## DISCUSSION

### *1. Nature of enzyme* —

Inorganic polyphosphatase of the frog had an acid pH optimum in the range of pH 3.9–4.4; a neutral pH optimum of 7.2–7.7; and no alkaline optimum (figs. 1, 2). It did not hydrolyse either glycerophosphate (at pH 3.9) or yolk phosphoprotein (in the duodenum of the stage 24 tadpole). The acid IPP-ase was not inactivated by acetate, even below pH 3.4. Both acid and neutral IPP-ase were activated by $Mg^{++}$ and inactive without it in tissue brei. Activation properties were altered by histochemical procedures (Berg, '55).

The few inorganic polyphosphatases described in metazoa since Kitasato's discovery in 1928 (a, b) have properties consistent with the above (Neuberg and Fischer, '38; Barnabei et al., '50; Gustafson and Hasselberg, '51; Mattenheimer, '51). There has been no prior report of IPP-ase in vertebrate embryos, nor have both the acid and neutral IPP-ase been shown to be simultaneously present in any tissue.

*2. Enzyme activity in embryonic development—*

In stages 15 and 16 of embryonic development acid IPP-ase was relatively abundant and was equally distributed in mesectoderm and yolk. By stage 25 it had approximately doubled its concentration in the mesectoderm without changing significantly in the yolk. This pattern of slow change was in sharp contrast to the behaviour of alkaline phosphatase which was much more concentrated in the mesectoderm than in the endoderm and, in addition, increased 7 to 10 fold in each fraction between stage 15–16 and stage 19. When the GP-ase activity of the two fractions was summed up and measured per embryo rather than per milligram of dry tissue, the activity doubled between stages 15 and 17 and increased 4 fold by stage 19. This same exponential rate of increase was found by Krugelis ('50) in *Xenopus*, where it continued from gastrulation to hatching.

The developmental changes in alkaline GP-ase in embryos were also followed by histochemical methods. The distribution found was the same as that given by Osawa ('51) for the frog, *Rhacophorus*, and agreed with Brachet's ('46) description of *Xenopus*. The strongly staining mesenchyme of organ primordia could account for most of the rise of GP-ase found microchemically in the embryonic mesectodermal fraction (table 3). The yolk fraction enzyme (table 3) was concentrated in the developing epithelial cells (pharynx and liver diverticulum), while the cells inside the yolk mass had low stainability. In relating these regional patterns to the analytical data on fractions, it became evident that the bulk of GP-ase activity found in whole-embryo homogenates came from mesenchyme.

*3. Enzyme activity and organogenesis of the duodenum —*

In the course of organogenesis of the duodenum, acid IPP-ase followed a pattern of gradual increase; the activity leveled off when the organ lost its yolk, and then remained constant into adult life. The alkaline GP-ase, on the other hand, remained low until the loss of yolk, and then rose abruptly with the establishment of function. Thus, the burst of alkaline GP-ase activity which was demonstrated by Moog ('50, '51, '52a, b) in the mouse and chick, was also present in the tadpole at the onset of herbivorous digestion. In the tadpole, as in the chick, there was only one peak of activity, correlated with the disappearance of yolk. This change in the metabolism of endoderm in the larve had little effect on IPP-ase activity. The later differences in enzymatic activities, occurring during the transition from larva to adult, had to be reinterpreted in the light of the histochemical data. The larval duodenum was practically a monocellular layer of enzyme-bearing mucosa, while in the adult the activity reading was diluted by the weight of many layers of enzyme-free tissue. Consequently, the biochemically measured decrease in GP-ase activity per unit dry weight, as well as the apparent constancy of IPP-ase activity between the two stages, was misleading. The epithelial cells contained more IPP-ase in the adult than in the larva, and probably more GP-ase.

In spite of the great variability in enzyme content among individuals the adult duodenum maintained a constant ratio of acid IPP-ase to alkaline GP-ase. This suggested a functional correlation within the cell between the two enzymes. In the duodenum of the premetamorphic tadpole, the ratio of IPP-ase to GP-ase was double that in the adult, indicating a physiological difference between tadpole and adult digestive epithelium.

*4. Histochemistry of alkaline glycerophosphatase* —

The distribution of alkaline GP-ase in *Rana pipiens* tadpoles and adults tallied with its distribution in *R. temporaria* adults, as given by Arvy and Gabe ('53). The intracellular distribution of GP-ase in the intestinal mucosa was in accord with that observed by Emmel ('45), who noted that the frog was exceptional in having no GP-ase in the "Golgi zone" of cytoplasm of its intestinal mucosa. A phosphatase-rich lining in the tadpole capillaries has been previously reported by Junqueira ('50). The strong stain shown in tadpole muscle was not the rule in amphibian larvae, since Karczmar and Berg ('51) found a low GP-ase stain in the muscle of salamander larvae. The goblet cells of a salamander intestine, unlike those of the tadpole, had GP-ase in the mucosa.

In the body surfaces of the amphibian, GP-ase appeared as a constant component of the lining of portions of the urinogenital tract, the lungs and the midgut. The stain varied from species to species in the lining of the circulatory system (Arvy and Gabe, '53). GP-ase was absent from the lining of the foregut, hindgut, and, in this experimental series, from the lining of the pharynx, opercular cavity and gills.

5. *Anatomical distribution of IPP-ase in relation to GP-ase* —

IPP-ase was found to cover much more body surface than alkaline GP-ase. Beginning with the nares, where IPP-ase rich cells were in contact with the environment while GP-ase was restricted to the deepest cell layer, acid IPP-ase alone was present in the continuous epithelial lining of the water-respiratory area and foregut. Similarly, IPP-ase alone lined the hindgut. It may be pertinent to note, that in the newly hatched minnow IPP-ase alone lined the yolk sac (Berg and Creaser, '53).

Acid IPP-ase was contained in the same cells as alkaline GP-ase in the nephron, small intestine and tongue. In all three cases it was located in secretory (or absorbent) epithelia where active surface transfer took place.

Acid IPP-ase was absent from muscular, neural and vascular elements, which had a high alkaline GP-ase content in the tadpole. IPP-ase was also absent from skeletal elements and mesenchymal organ primordia, which were rich in GP-ase.

Thus, IPP-ase was primarily an enzyme of epithelia. It was coupled with GP-ase only in secretory or absorbing cells. A generalization of its physiological significance would, however, have to take into account its presence in the specialized elements of pancreas, stomach and liver, and its appearance in early embryogenesis.

The intracellular distribution of IPP-ase followed two patterns. The stain was either present in a restricted zone of cytoplasm or it filled the cytoplasm. In two cases, that of the brush border epithelium and of the duodenal columnar epithelium, the staining zone corresponded to the area of the cell in which mitochondria were densely packed.

6. *Physiological significance* —

In considering the physiological function of inorganic polyphosphatase, one comes against the problem of the availability of its substrate in the vertebrate cell. Techniques, which served to demonstrate polyphosphate in yeast (Liebermann, 1888, after Ebel, '52b), other fungi (Mann, '44), bacteria and algae (Ebel, '52f), were not adequate for the demonstration of this substrate in animals (Ebel, '52d, e); but even the modern chromatographic techniques of Ebel are of limited sensitivity. The negative findings can only be interpreted to mean that animals do not accumulate deposits of polyphosphate. On the other hand, energy metabolism involves the release of phosphoric acid, and nascent phosphate may polymerize into polyphosphate. Inorganic polyphosphatase may, therefore, function in the vertebrate to prevent the accumulation of polyphosphate of metabolic origin. Since polyphosphate is a protein denaturing agent (Balareff, '28) and one of the most potent chelating agents (Gosselin et al., '53), polyphosphatase activity assumes the character of a detoxifying function. This hypothesis would account for the presence of polyphosphatase as a companion enzyme in the cells in which alkaline phosphatase releases large amounts of phosphate.

If a vertebrate cell does not synthesize polyphosphate, it may still come into contact with polyphosphate of exogenous origin. In that context, the detoxifying function of polyphosphatase has already been established by pharmacological experiments. Doses of polyphosphate which are lethal on parenteral administration become inocuous when administered orally, and this loss of toxicity is correlated with the presence of polyphosphatase in the digestive tract (Barnabei et al., '50). This detoxification of orally administered polyphosphates may be of importance in normal vertebrate physiology, when polyphosphate-bearing microorganisms are ingested with food. The very presence of this digestive barrier, however, excludes the possibility that exogenous polyphosphates find their way into the circulatory system; and yet parenterally administered linear polyphosphate is rapidly broken down (Gosselin et al., '52). Thus the pharmacological findings are in agreement with the anatomical distribution of polyphosphatase as shown histochemically; and cannot be accounted for on the basis of a substrate found in the external environment only.

A fundamental metabolic function was recently ascribed to inorganic polyphosphate by Rothstein and Meier ('51). These authors regard polyphosphate in trace amounts as an integral component of the yeast cell membrane, where polyphosphate inclusions serve to bind metallic ions, and are thus concerned in enzymatic membrane transfer of carbohydrates. It is perhaps permissible to extend this hypothesis, and postulate that cytoplasmic polyphosphatase functions in regulating the level of the membrane-bound polyphosphate, and that this polyphosphate-polyphosphatase system is present in the metazoa. If the pattern of polyphosphatase staining in the tadpole reflects the distribution of such a polyphosphate-polyphosphatase system, then this system would appear to function in the areas where the vertebrate organism takes up solutes from its liquid environment.

## SUMMARY

1. Enzymes hydrolysing inorganic polyphosphate were shown to be present in the embryo, larva and adult of *Rana pipiens*. These inorganic polyphosphatases (IPP-ases) are also known as "metaphosphatases." Their substrate, derived from Graham's salt and sold as "metaphosphate" is a linear polymer with $n > 3$.

2. Assays of IPP-ase activity in homogenates of adult duodenum and whole stage 18 tadpoles yielded optima near pH 4.2 and pH 7.5. IPP-ase in homogenates required Mg as an activator (0.004 M) and was not inhibited by inorganic polyphosphate ($10^{-2}$ M with respect to P).

3. In comparative assays of mesectodermal and endodermal fractions of stage 15 and 16 embryos, acid IPP-ase activity per unit dry weight was relatively high and uniformly distributed; only mesectoderm had a significant increase in stage 19. GP-ase, by contrast, showed a strong preponderance in mesoderm and an exponential increase in activity from very low in stage 15 to higher than IPP-ase in stage 19.

4. In the developing duodenum, acid IPP-ase activity per unit dry weight increased slowly until the beginning of function in the tadpole (stage 25) and did not change afterward. Alkaline GP-ase activity per unit dry weight was negligible in the stage 24 tadpole, high in stage 25, and half as high in the adult as in the stage 25 tadpole. The ratio of acid IPP-ase to alkaline GP-ase in homogenates of adult duodenum was constant.

5. Histochemical distribution of acid IPP-ase was mapped in representative adult organs and compared with alkaline GP-ase. Organs containing only IPP-ase (stomach), only GP-ase (heart), and both were found. In duodenum, mesonephros and tongue the two enzymes were distributed in the same cells.

6. Histochemical distribution of acid IPP-ase was mapped in the premetamorphic tadpole (stage 25) and compared with alkaline GP-ase. Acid IPP-ase was an enzyme of epithelia. It covered the entire water-respiratory and digestive areas from buccal and opercular cavities through rectum, and was present in chief glands, liver, pancreas, and pronephric convoluted tubules. It overlapped with GP-ase in midgut and pronephros, but differed in intracellular distribution.

7. Function of IPP-ase in the vertebrate is discussed.

## ACKNOWLEDGMENTS

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PLATE

## PLATE 1

### Premetamorphic *Rana pipiens* Tadpole (Stage 25)

3 Acid IPP-ase in a cross-section through the otic region and heart. Stain in the epithelium of pharynx, gills and opercular cavity. Other black sites are melanin. Alcohol-acetone fixation. 7 hours of incubation, 30 ×.

4 Alkaline GP-ase in the same region as figure 3. Stain in neural and muscular elements, core of gills and heart ventricle. Alcohol-acetone fixation. 10 hours of incubation. 30 ×.

5 Acid IPP-ase in a cross-section through region of pronephros. Stain in proximal convoluted tubules, liver, oesophagus and intestine. Other structures show only melanin or the eosin counterstain. Note enzyme-free collecting tubule. Freeze-substitution fixation 5½ hours of incubation. 40 ×.

6 Acid IPP-ase in a cross-section through the mid-body region. Stain in stomach, pancreas and oesophagus. Other structures show only melanin or the eosin counterstain. Note unstained lung. Freeze-substitution fixation. Five and a half hours of incubation. 40 ×.

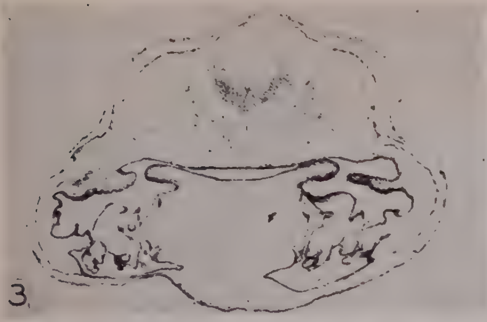
7 Acid IPP-ase in a proximal convoluted tubule from preparation shown in figure 5. Stain in cytoplasm, no stain in nuclei and brush border region. Freeze-substitution fixation. Five and a half hours of incubation. Eosin counterstain. 200 ×

8. Acid IPP-ase in a segment of duodenum wall. Lumen is in upper left, coelom in lower right. Note absence of stain from cuticular border and from nuclei. Freeze-substitution fixation. Five and a half hours of incubation. Eosin counterstain. 600 ×.

9 Alkaline GP-ase in a segment of duodenum. Lumen is on left, coelom on right. Stain in cuticular border. Alcohol-acetone fixation. Five hours of incubation. Eosin counterstain. 600 ×.

10 Acid IPP-ase in the stomach wall from specimen shown in figure 6. Stain in cytoplasm of mucous cells at lumen and in cytoplasm of chief cells. Freeze-substitution fixation. Five and a half hours of incubation. Eosin counterstain. 600 ×.

11 Alkaline GP-ase in a cross-section through mid-body region. Stain in neural elements, skeletal muscle, gall bladder, liver and intestine. Note absence of stain from stomach (extreme left) and oesophagus. Alcohol-acetone fixation. Five hours of incubation. 30 ×.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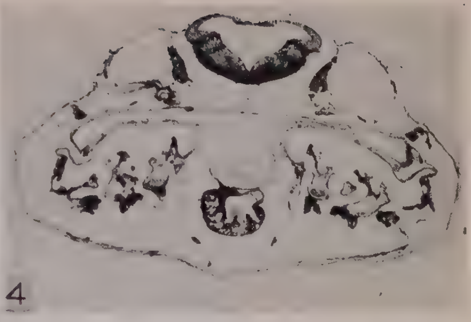
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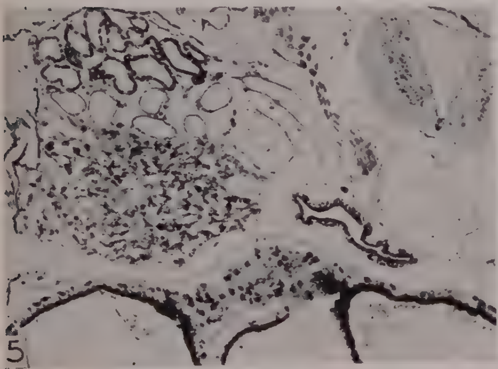
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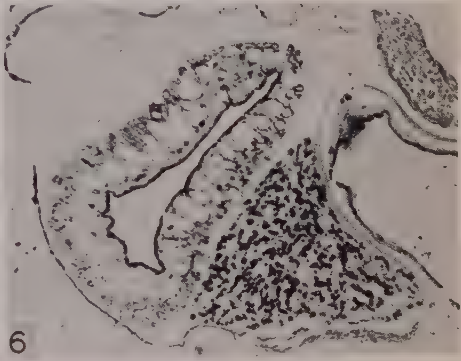
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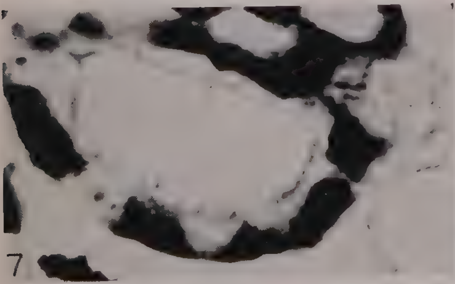
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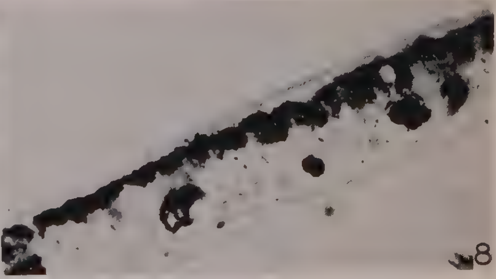
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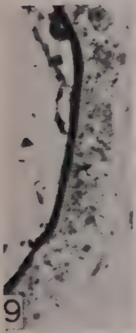
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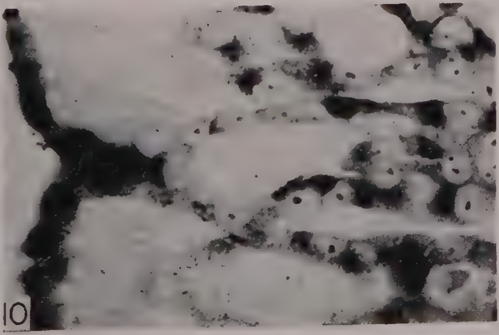
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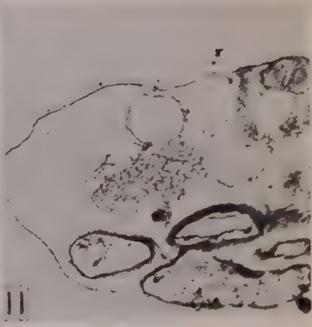
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# THE OSMOTIC WORK OF SALIVARY SECRETION IN THE DOG

A. S. V. BURGEN
*Department of Physiology, McGill University, Montreal, P. Q., Canada*

## INTRODUCTION

Since the earliest work on salivary secretion it has been recognized that the concentration of some of the more important solutes were greater at high rates of secretion than at slower rates (Babkin, '44). This relationship is particularly evident with the predominant cation, sodium and anion, bicarbonate (Gregersen and Ingalls, '31; Langstroth, McRae and Stavraky, '38; Thaysen, Thorn and Schwartz, '54). The rise in solute concentration must mean that the total osmotic pressure of saliva rises with rate of secretion. There have been many estimations of the freezing point depression of saliva (Babkin, '44) which agree in finding that parotid saliva with which we are primarily concerned is always hypotonic to the plasma, but we have been unable to find any systematic study of the osmotic pressure of saliva as a function of rate of secretion.

We have had three objectives in this investigation. In the first place it seemed desirable to establish the limits of osmotic pressure found in the saliva. Secondly, we wished to test whether the rise in concentration of solutes with increased secretion rate might be due to a saturation of the gland capacity for performing external osmotic work. In this case it would be expected that with increasing rate of secretion the osmotic work of secretion would reach a maximum and remain at that level. In the third place it seemed unlikely that the duct system of the salivary gland could be completely impermeable to water molecules, and hence as a preliminary to measuring water reabsorption by the ducts we hoped to define the magnitude of the osmotic gradient involved.

In the present work, the osmotic pressure of the parotid saliva of the dog has been studied. The rate of secretion has been varied by changing the rate of stimulation with maximal volleys of the sectioned *post*-ganglionic nerve supply. Parotid secretion was chosen for study because the acini contain only a single cell type, the accessibility of the post-ganglionic nerve supply simplifies interpretation, and because with a secretion consisting almost exclusively of water and inorganic salts, it is reasonable to assume that the work of formation of secretion consists almost entirely of concentration work and only to a minor extent of chemosynthetic work.

In this paper the term *osmotic work* has been used in the strict sense as applying to that form of concentration work which increases the chemical potential of *water* regardless of the detailed concentration changes which have occurred in the solutes.

## METHODS

Dogs were anaesthetized with a 1:10 chloralose-urethane mixture I.V. The auriculotemporal nerve was exposed at the base of the skull by an incision on the medial side of the angle of the lower jaw, followed by retraction of the pharynx and division of the medial and lateral pterygoid muscles. The nerve was dissected up to its point of emergence from the skull, ligated and cut proximally. The situation of the nerve close against the skull at the apex of a narrow cleft, makes the use of specially designed submersible stimulating electrodes imperative. This problem was solved by the use of pore electrodes. The electrode was composed of two chlorided silver rings set in separate slots in a block of Lucite. A hole slightly larger than the diameter of the nerve was bored in the Lucite to pass through the axis of the silver rings. When the nerve was drawn through this hole it made contact with the silver rings through a 0.5 mm ring of saline. The parotid duct was exposed in the cheek and cannulated with a cannula made from a short length of 18 or 20 gauge hypodermic needle and extended by a 2–3 cm length of polyethylene tubing. The resistance of this cannula to the flow of saliva was less than 1 cm $H_2O$ at the maximum rate of secretion and its dead space was less than one drop. Secretion of saliva at various rates was produced by supramaximal stimulation of the nerve with rectangular pulses of 0.5–2 msec duration at frequencies of 0.5–50 cycles/sec. After the first 4 or 5 drops had been discarded saliva was collected for a timed period in a tared screw cap tube. The rate of secretion was determined by weighing the samples. An interval of 4 minutes was allowed between each collection period. The rate of saliva secretion was checked by timing drops with a stopwatch in order to be sure that secretion was proceeding at a constant rate.

The freezing points of saliva and heparinised plasma were determined in duplicate with a copper-constantan thermocouple to an accuracy of 0.01°C. by a method similar to that described by Conway and McCormack ('53). At the end of the experiment the gland was dissected out and weighed and the flow rate could then be expressed as mg/gm gland/minute, denoted by the symbol V. Other symbols are used as follows: $\pi p =$ plasma osmotic pressure; $\pi s =$ saliva osmotic pressure; $\Delta\pi = \pi p - \pi s$; $W_\pi =$ the minimum work expended per litre of saliva in increasing the chemical potential of water in the plasma to that in the saliva; $E_\pi =$ the minimum work of water transfer expended by the gland to form saliva at the secreted rate and concentration.

## RESULTS

Complete curves relating osmotic pressure of the parotid saliva to the rate of flow have been obtained in 6 dogs. There was little variation in the results from different animals. From flow rates of the order of 40 mg/gm/min. upwards there was a steady increase in the osmotic pressure of the saliva with increase in the rate of secretion. At rates below 40 mg/gm/min. the osmolyte concentration either remained constant or showed a slight rise if very slow rates were attained.

Figure 1 shows a representative experiment in which the minimum osmolyte concentration was 74 m Osmols (at V = 32.6) and the maximum was 219 m Osmols at the maximum rate of secretion (V = 592). The maximum osmotic difference from the plasma (osmolyte concentration 299 m Osmols) corresponds to an osmotic pressure of 5.69 atmospheres. In table 1 it may be seen that the mean figures for the 6 experiments were minimum osmolar concentration (π min) = 82 ± 8.1 m Osmols; maximum osmolar concentration (π max) = 232 ± 10.8 m Osmols; and maximum osmotic pressure difference between saliva and plasma (Δπ max) = 5.89 ± 0.22 atmospheres.

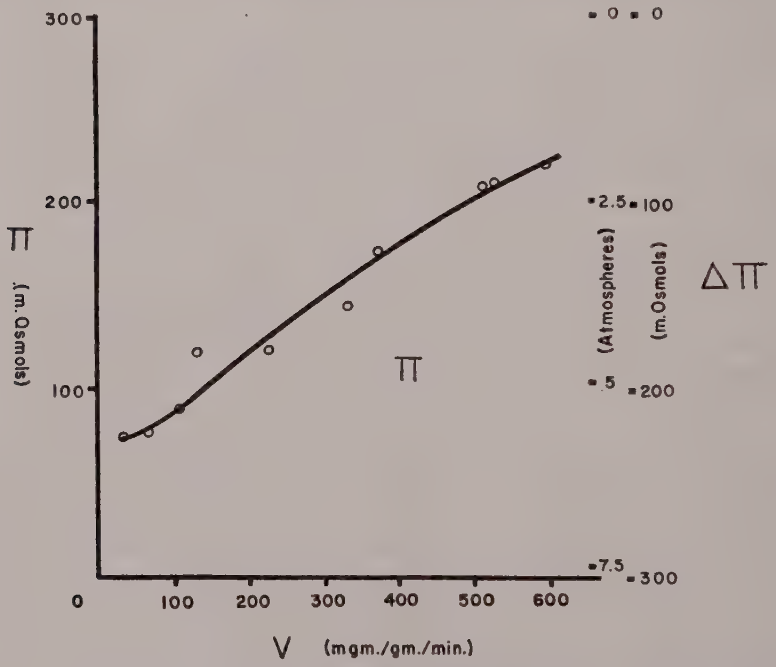

Fig. 1 Osmolyte concentration of saliva (left hand scale) and osmotic pressure difference between saliva and blood (right hand scale) versus rate of saliva secretion.

These results accord fairly well with calculations based on the known sodium and potassium concentrations of the saliva.

Now the minimum work of transferring 1 litre of water into an isotonic saliva is given by the expression

$$\text{Work}_{\text{litre } H_2O} = \underset{T_1P_0}{\mu^{o\prime}} - \underset{T_1P_0}{\mu^{o\prime\prime}} = RT\,(\pi p - \pi s) \qquad (1)$$

where $\underset{T_1P_0}{\mu^{o\prime}}$ , $\underset{T_1P_0}{\mu^{o\prime\prime}}$ are the chemical potentials of water in the saliva, and plasma respectively, and R is the gas constant, T the absolute temperature, and $\pi p$ and $\pi s$ the osmotic pressures of plasma and saliva. If the units are chosen correctly, i.e., $R = 1.986$ cals/°C./mol, T in absolute degrees, and $\pi p$, $\pi s$ in Osmols/L the work will be given in calories per litre.

TABLE 1

*The mean osmotic pressure and osmotic work in 6 dogs*

| | MEAN ± S.E. | RANGE | UNITS |
|---|---|---|---|
| $\pi_s$ min. | 82 ± 8.1 | 48–103 | m. Osmols. |
| $\pi_s$ max. | 232 ± 10.8 | 198–263 | m. Osmols. |
| $\Delta\pi$ min. | 1.88 ± 0.27 | 1.17–2.46 | Atmospheres |
| $\Delta\pi$ max. | 5.89 ± 0.22 | 5.35–6.27 | Atmospheres |
| $W_\pi$ max. | 10.5 ± 7.0 | 80–124 | cals./litre |
| $E_\pi$ max. | 13.2* ± 2.7 | 6.7–23.6 | millicals./gm/min. |
| $E_\pi$ at V max. | 6.4* ± 1.7 | 2.4–13.0 | millicals./gm/min. |
| $\dfrac{E_\pi \text{ at V max.}}{E_\pi \text{ max.}} \times 100$ | 49 ± 3.7 | 36–55 | % |
| V at which $E_\pi =$ max. | 47 ± 2.8 | 36–53 | % of max. flow rate |
| Max. osmolyte reabsorption | 48 ± 5.4 | 32–66 | μ Os./gm/min. |
| Max. free water secretion | 160 ± 18 | 107–220 | mg/gm/min. |

* Geometric means and errors.

In each litre of saliva there is only $(1 - \frac{\pi s}{\pi p})$ litres of transferred water, so that the minimum osmotic work of formation of *1 litre of saliva* is

$$W_{\pi\,\min} = RT\,(\pi p - \pi s)\,(1 - \frac{\pi s}{\pi p}) \qquad (2)$$

$$= \frac{RT}{\pi p}(\pi p - \pi s)^2 \text{ cals/litre of saliva}$$

Further the work rate of the gland (or minimum energy expenditure) in secreting this saliva is simply obtained by multiplying $W_{\pi\,\min}$ by the rate of secretion in litres/gm gland/min $\left(=\frac{V \times 10^{-6}}{\text{Specific gravity of saliva}}\right)$[1] thus

$$E_{\pi\,\min} = \frac{V.RT}{\pi p.SG}(\pi p - \pi s)^2 \times 10^{-6}\ \text{cals/gm/min.} \qquad (3)$$

The application of equation (2) to the results of the experiment presented in figure 1 are shown in figure 2 in which it can be seen that $W_\pi$ fell from 105 cals/L at the lowest flowrate to 14 cals/L at the highest flowrate; in cases where the osmotic pressure went through a minimum at low flowrates there was a corresponding maximum in the osmotic work curve. Over the whole series the maximum value of $W_\pi$ was 105 ± 7.0 cals/L and the minimum 12.1 ± 3.8 cals/L.

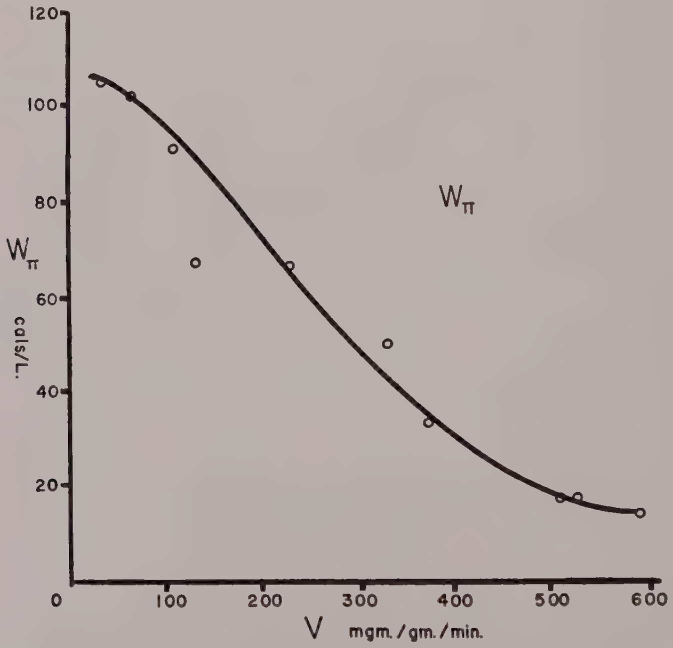

Fig. 2 Minimum work of formation of saliva versus rate of saliva secretion.

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[1] Little error is introduced if the specific gravity of saliva is taken as constant at 1.01.

When we consider the application of equation (3) the results are somewhat surprising. $E_\pi$ in all the experiments rose with rate of flow to a maximum at about half maximal flowrates and then declined to about half the maximum value at the highest flowrate. Figure 3 shows the result in the present case; the work rose from the origin to a maximum of

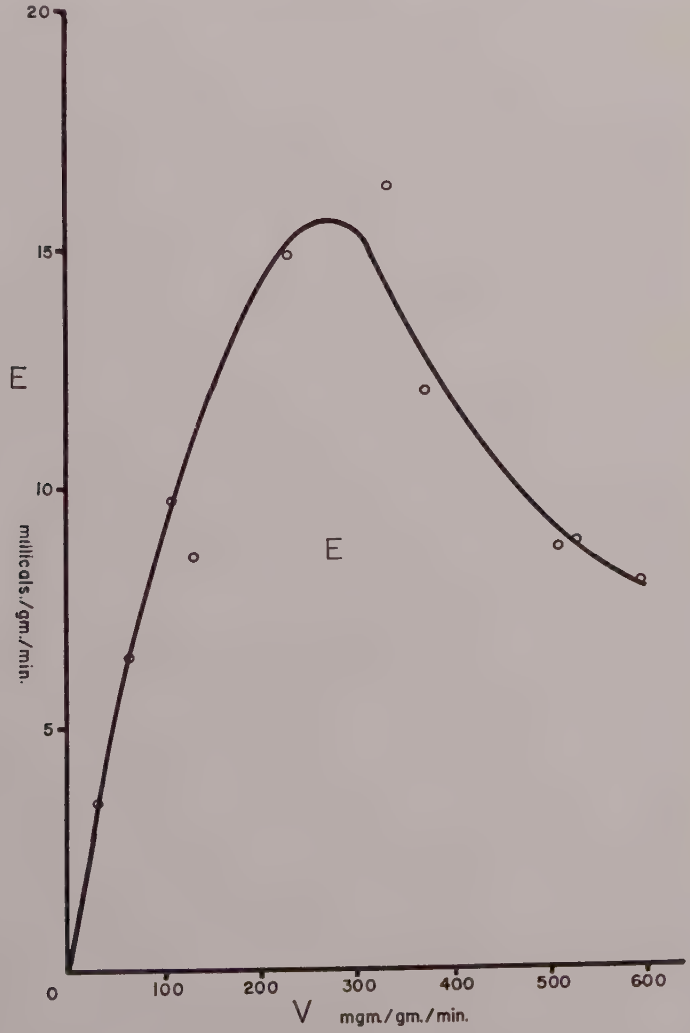

Fig. 3 Minimum work of secretion per minute versus rate of saliva secretion.

15.7 millicals/gm/min. at V = 280 mg/gm/min. and fell again to 8.7 millicals/gm/min. at the maximum flow where V = 594. The mean maximum of $E_\pi$ for all six experiments was 13.2 ± 2.7 millicals/gm/min. at 47 ± 2.8% of the maximum flowrate and fell to 6.4 ± 1.7 millicals/gm/min.; an average of only 49 ± 3.7% of the peak work at the maximum flowrate.

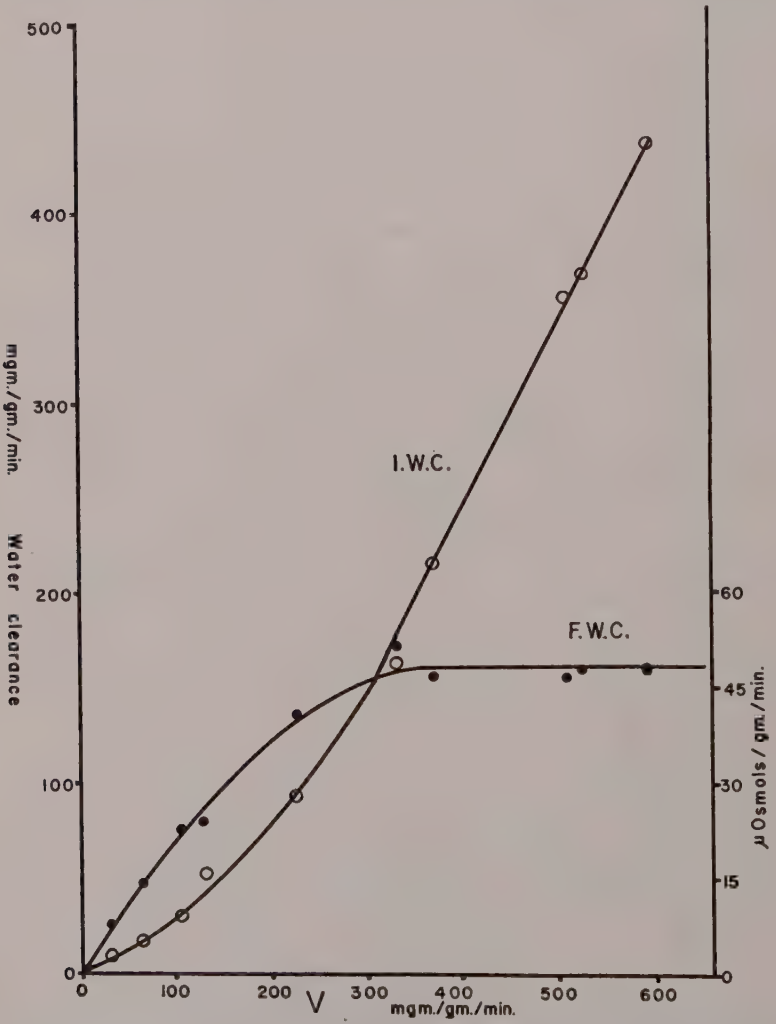

Fig. 4 Free and isotonic water secretions (left hand scale) and electrolyte reabsorption (right hand scale) versus rate of saliva secretion.

The calculation of osmotic work assumes that water is being actively transferred against a gradient, the amount of water being transferred is given by V $(1-\frac{\pi s}{\pi p})$ mg/gm/min. and may be called the Free water clearance, the rest of the salivary water requires no *osmotic* work and may be called Isotonic water clearance.

$$\text{Free water clearance (FWC)} = V\left(1-\frac{\pi s}{\pi p}\right)\ \text{mg/gm/min.} \qquad (4)$$

$$\text{Isotonic water clearance (IWC)} = \frac{V\pi s}{d\mu}\ \text{mg/gm/min.} \qquad (5)$$

Figure 4 shows that the FWC reaches a maximum at about V = 400 and remains at this level up to the maximum flowrate. The maximum FWC in this case was 160 mg/gm/min. and in the series was 160 ± 18 mg/gm/min. The IWC, on the other hand, increases more steeply as the flowrate increases.

## DISCUSSION

The results presented in this paper show that there is a regular relationship between the rate of secretion of saliva and its osmolar concentration.

One obvious explanation of the rise in concentration with increasing rate of secretion could be that the secretion has the minimum osmolyte concentration (or maximum water concentration) compatible with the glands' ability to do osmotic work. If this were the case we should expect to find that the calculated osmotic work rate ($E_\pi$) would show evidence of saturation, i.e. it should asymptote to a maximum level with increasing rates of secretion. On the contrary the experimental results show a clear maximum work rate at submaximal flowrates with a decline in work rate at higher rates of secretion.

No data are available on the respiration rate of the intact parotid gland, however, the oxygen consumption of the dog submaxillary gland has been measured by Barcroft ('38) who found in the resting state an utilisation of 0.03 $cm^3$ oxygen/gm/min. and in a state of maximum activity 0.1 $cm^3$ oxygen/

gm/min. Using the approximate equivalence of 1 cm$^3$ $O_2$ = 5 calories, this would mean a maximum respiratory energy production of about 500 millicalories/gm/min. If we assume the respiration of the parotid gland is about equally active, the external osmotic work of secretion amounts at most to about 4% of the available energy.

The resolution of the saliva water into free water and isotonic water by equations 4 and 5 reveals the interesting relation that the FWC reaches a maximum rate at about two-thirds the maximum rate of secretion and maintains this rate in the face of increase in rate of secretion, so that the increase in flowrate above V = 400 is entirely due to isotonic water clearance (fig. 4). Whilst this division of the water into two fractions is just a convenient hypothesis it may indeed mean that the cell is capable of secreting water *qua* water at one series of loci in the mosaic of the cell membrane and an isotonic solution at another series of loci.

There is, however, another process capable of yielding the same result, namely secretion of an isotonic solution with reabsorption of solute up to a maximum rate (analogous to the behaviour of glucose in the kidney at plasma levels above the "threshold") — the free water excretion curve could be supplied with a new ordinate scale of μ Osmols/gm/minute reabsorbed (fig. 4, right hand scale) — the maximum FWC of 160 mg/gm/min. would correspond to a maximum solute reabsorption of 48 μ Osmols/gm/minute. It should be noted that the work of reabsorption of the solute is slightly different from the osmotic work and is given by

$$W_R = RT\,(\pi p - \pi s)\,\ln\frac{\pi p}{\pi s} \qquad (6)$$

$$E_R = \frac{VRT}{SG}(\pi p - \pi s)\,\ln\frac{\pi p}{\pi s} \times 10^{-6} \qquad (7)$$

Calculation shows that $E_R$ bears a similar relationship to rate of flow as does $E_\pi$ but the maximum work is somewhat higher, 17.6 ± 4.0 millicalories/gm/min. In considering this second mechanism we must clearly give thought to the possibility that the solute reabsorption may occur in the gland ducts. Now the total duct volume in sections of dog parotid fixed in Formol-Sublimate was 5–10% of the gland volume so that if all the solute reabsorption occurred in the ducts, the energy required would be 176–352 millicalories/gm duct tissue/min. If the metabolism of the ducts is similar to that of the gland as a whole this represents a large proportion of the available energy, but not large enough to make it possible to exclude such a mechanism.

In all the discussion so far we have been evaluating the secretion as it appears in Stensen's duct; this, however, may not represent accurately the secretion as elaborated in the acini by the primary secreting process. Leaving aside the possibility that the ducts may contribute specifically to the secretion, it is most unlikely that they are completely impermeable to water. There must therefore be some reabsorption of water from the saliva along the osmotic gradient between the hypotonic saliva and the plasma. If this reabsorption were to represent an appreciable fraction of the secreted water, we would be seriously in error in drawing conclusions about the secretory process from the nature of the external secretion. However, such a reabsorption of free water would be greatest at low flow rates owing to the greater osmotic gradient, and would also represent a larger fraction of the total flow at these rates. This would tend to raise the peak work rate slightly and shift it to a lower flow rate, but it would not affect the general conclusions we have reached. Such a reabsorption might account for the levelling off or rise in salivary osmolyte concentration that occurs at very low flow rates. Preliminary estimates of the rate of osmotic reabsorption suggest an order of 20–30 mg $H_2O$/Osmol gradient/g/min.

This estimate was obtained by the study of non-electrolyte secretion in the saliva. With some non-electrolytes (urea, N methyl urea, thiourea) in which secretion appears to be passive, that is, the saliva/plasma ratio is independent of the plasma level, at very low rates of secretion the concentration in the saliva may rise *above* the plasma level. Since the solute is behaving passively, this concentration must be due to reabsorption of solvent. From this data and the osmotic pressures of saliva and plasma, the above estimate of osmotic water reabsorption was arrived at. Full details of this work will be published elsewhere.

This rate of water reabsorption would raise the peak work rate in the example given by about 6% and shift the peak a few per cent to the left. It seems probable that for rates of flow above about 70 mg/gm/min. the error in assuming that the duct secretion accurately reflects the acinar secretion may be small.

## CONCLUSIONS

1. The freezing point depression of dog parotid saliva has been studied as a function of the rate of secretion.
2. The osmolyte concentration of the saliva increased with the rate of secretion from 82 ± 8.1 m Osmols at the lowest rates of secretion up to 232 ± 10.8 m Osmols at the maximal rate.
3. The osmotic work of secretion reached a maximum of 13.2 ± 2.7 millicals/gm/min. at about half maximal secretory rates and declined with further increase in rate of secretion to 6.4 ± 1.7 millicals/gm/min. at the maximal rate.
4. It is therefore unlikely that the rise in osmolyte concentration in the saliva with increasing rates of flow is due to saturation of the glands capacity for osmotic work.
5. The role of osmotic back absorption of water in the ducts is discussed.

## ACKNOWLEDGMENTS

I am greatly indebted to Miss Nora Pedley for her expert assistance and the National Research Council of Canada for financial support.

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# COMMENTS
# AND
# COMMUNICATIONS

Comments relating to articles which have recently appeared in the Journal of Cellular and Comparative Physiology and brief descriptions of important observations will be published promptly in this Section. Preliminary announcements of material which will be presented later in more extensive form are not desired. Communications should not in general exceed 700 words.

## AN INSULIN EFFECT ON SPONGE METABOLISM

EDWIN E. GORDON,[1] MELVIN SPIEGEL[2]
AND CLAUDE A. VILLEE

*Departments of Biological Chemistry and Obstetrics, Harvard Medical School, Boston, Massachusetts*

*Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena, California*

*The Marine Biological Laboratory, Woods Hole, Massachusetts*

ONE FIGURE

It was shown by Krebs and Eggleston ('38) using a pigeon muscle mince in the presence of boiled muscle extract and various intermediates of the citric acid cycle, that insulin could effect an increase in oxygen uptake. This work was subsequently confirmed by other workers (Shorr and Barker, '39; Stadie et. al., '40; Stare and Baumann, '40). We have been able to demonstrate this action of insulin on an invertebrate — the sponge, *Microciona prolifera.*

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[1] Winthrop-Stearns Research Fellow in Obstetrics, Harvard Medical School and the Boston Lying-in Hospital, Boston, Massachusetts.

[2] Research Fellow in Embryology. Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena, California.

## MATERIALS AND METHODS

The red, colonial sponge, *Microciona prolifera*, was obtained from the supply department of the Marine Biological Laboratory at Woods Hole, Massachusetts. The sponges were maintained in constantly changing sea water and were used in the experiments within two weeks of the time of collection. Apical portions of the finger-like projections of the sponge were cut into 2–5 mm pieces, allowed to dry for approximately two minutes on absorbent paper and weighed on a torsion balance. Care was taken to avoid discolored areas. About 200 mg of sponge were placed in the main chamber of each Warburg vessel in 3 ml of incubation medium. Either potassium pyruvate, glucose, or sodium citrate in filtered sea water served as substrate in concentrations of 1 mM/L, 5.6 mM/L and 1 mM/L, respectively. Experiments were also performed without added substrate. Four units of insulin (Iletin, Lilly) containing 0.2% phenol as a preservative were added to each of the appropriate vessels. This insulin was prepared from zinc insulin crystals. As controls, distilled water or 0.2% phenol was added in place of insulin. Sodium hydroxide was present in the center well of the vessels to absorb carbon dioxide. The vessels were incubated for two or three hours at 37.2°C. in an air atmosphere. At the end of this period, aliquots of the medium were removed for chemical determination.

Glucose was determined by the method of Nelson ('44), keto acids by combination with 2-4,dinitrophenylhydrazine (Friedemann and Haugen, '43) and citric acid by a slight modification of the Natelson, Pincus and Lugovoy method ('48).

Sponge homogenates were prepared by grinding about 4 gm of cut sponge in 10 ml of ice cold sea water with a Teflon homogenizer. The suspension was centrifuged for 10 minutes at 700 × g. The inert silicious material and cellular debris were then separated from the translucent, homogeneous, orange supernatant. The supernatant was then incubated as described above.

A disaggregated cell suspension was prepared by squeezing pieces of sponge through bolting silk into Van't Hoff calcium-free artificial sea water. The preparation was washed twice, resuspended in filtered sea water and pipetted into the Warburg vessels. This preparation consists of intact cells, but eliminates the inert spicules. Partial reaggregation of the cells occurred during the incubation period.

## RESULTS

The oxygen consumption of the sponge *Microciona prolifera* was consistently greater in the presence of insulin than in the control (table 1). Phenol in the concentrations present in the commercial insulin had no effect on the sponge respiration. Oxygen uptake was not affected either in the presence or absence of insulin by using different substrates (citrate, pyruvate, or glucose). Indeed, the sponges respired as well without added substrate. When glucose served as the substrate and phenol the control, the $Q_{O_2}$ ($\mu$l $O_2$/mg wet weight/hr.) in the absence of insulin was 0.123 ± .007 (mean ± SE) and in the presence of insulin 0.145 ± .008, with an average increase in the presence of insulin of 17.9%; $P < 0.01$ from paired t tests. The results of a typical experiment are presented graphically in figure 1. The insulin-induced increase in oxygen uptake is apparent at 15 minutes and continues through the experimental period. The rate of respiration begins to level off at the end of the second hour. No uptake by the tissues of glucose or citrate could be demonstrated. Sponges incubated in the presence of pyruvate showed a net production of keto acid; no insulin effect on this keto acid production could be detected.

TABLE 1

*The effect of insulin on M. prolifera respiration*

| EXPT. NO. | SUBSTRATE | $Q_{O_2}$ $\mu$l $O_2$ uptake/mg wet wt./hr. | | |
|---|---|---|---|---|
| | | *Control* | | *Insulin* |
| | | Distilled water | Phenol | |
| 1 | Glucose | | 0.139 | 0.154 |
| 2 | Glucose | 0.111 | 0.116 | 0.133 |
| 3 | Glucose | 0.099 | 0.100 | 0.119 |
| 4 | Glucose | 0.113 | 0.118 | 0.143 |
| 5 | Glucose | | 0.167 | 0.204 |
| 6 | Glucose | | 0.100 | 0.122 |
| 7 | Glucose | 0.118 | | 0.150 |
| 8 | Pyruvate | | 0.121 | 0.136 |
| 9 | Pyruvate | 0.126 | | 0.140 |
| 10 | Pyruvate | | 0.170 | 0.211 |
| 11 | Pyruvate | | 0.148 | 0.168 |
| 12 | None | 0.115 | | 0.149 |
| 13 | None | | 0.157 | 0.188 |

The values given are the mean of duplicate samples.

Homogenates and disaggregated cell suspensions utilized oxygen at a rate comparable to that of the whole tissue, but no insulin effect could be demonstrated with these preparations. No utilization of glucose was observed in the control or insulin incubated preparations.

In contrast to the consistently demonstrable insulin effect on respiration, neither thyroxine (100 $\mu$g per vessel) nor estradiol (3 $\mu$g per vessel) had an effect on the oxygen uptake or the utilization of glucose by the whole sponge.

## DISCUSSION

To our knowledge this is the first report of an effect of insulin on invertebrate metabolism. Previous studies (Villee et al., '50) showed no effect of insulin on glucose utilization or glycogen synthesis in tissues from a variety of higher invertebrates.

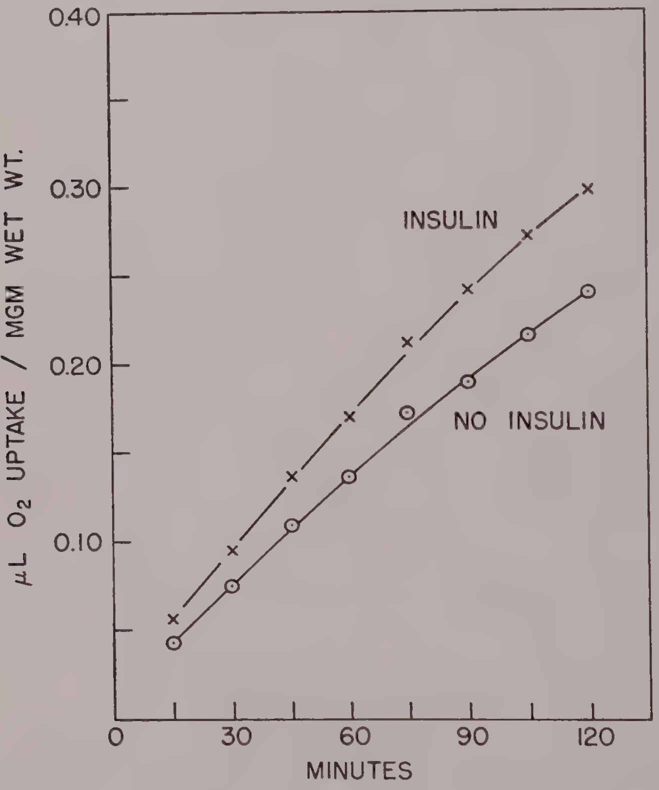

Fig. 1 $Q_{O_2}$ of *M. prolifera* in the presence of insulin and in distilled water control.

The sponge, *M. prolifera,* has a number of different cell types and a definite cytoarchitecture. In a sense, these were *in vivo* experiments. Except for the high temperature of incubation (37.2°), the sponge continued to metabolize in an external environment that was little different from its habitat in the sea.

In contrast to Krebs and Eggleston's experiments ('38) with pigeon muscle suspensions, the ratio of $O_2$ uptake in the presence of insulin to that of the control was approximately the same throughout the incubation period (fig. 1). If readings were continued for three or 4 hours, the $O_2$ uptake of both the control and insulin incubated tissues increased rather abruptly; this phenomenon most probably corresponds to the accelerated growth phase of the bacteria present in the filtered sea water.

No attempt was made to eliminate the possible non-specific protein effects of insulin.

The absence of an insulin effect on glucose utilization by the sponge suggests that the effect on oxygen uptake is the result of an action on one or more of the oxidative enzymes rather than an enhancement of glucose transfer across the cell wall or stimulation of the hexokinase system.

## SUMMARY

1. Insulin increased the oxygen uptake of the sponge, *M. prolifera* by an average of 17.9% over the controls.

2. The utilization of exogenous substrates (glucose, citrate, or pyruvate) by this preparation could not be demonstrated, either in the presence or absence of insulin.

3. Preliminary experiments with homogenates and disaggregated cells indicated that insulin had no effect on these preparations.

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# ELECTROLYTES IN THE TISSUES OF ELECTROPHORUS ELECTRICUS, L.

KEICA NISHIE AND E. J. HARRIS[1]
*Instituto de Biophysica, Rio de Janeiro*

In any discussion of the magnitude of the potentials existing between the interiors of cells and the external medium it is necessary to have knowledge of the composition of the material, in particular the internal and external concentrations of the mobile ions needs to be known. The present communication presents the results of analyses of tissue taken from Electrophorus Electricus and extends the data given by Davson and Lage ('53).

## METHODS

*Sodium and potassium.* Samples of tissue weighing about 0.5 gm were taken and dried overnight at 90°. The dry weight was determined and the samples were then ashed in a furnace at 500°. The ash was treated with water and made up to a volume having sufficient dilution to permit analysis by flame photometry.

Plasma was diluted and analyzed directly, blood cells were treated in the same way as tissue samples.

*Chloride.* Samples of tissue, or plasma, were wet ashed with a mixture of nitric acid, excess standard silver nitrate, and potassium permanganate, the latter was added in drops as necessary. The excess silver was determined by the Volhard method.

## RESULTS

*The plasma.* This was obtained by centrifugation of a blood sample withdrawn from the heart. The Na concentration was found to be 180 $\mu$eq/g in one fish, 184 in another and only 148 in a third. In the same plasmas the K concentrations were 5.8, 3.8 and 4.2 $\mu$eq/g respectively. In these determinations the error is estimated as $\pm 1\%$. The Cl concentration in each of two fish was 139 $\mu$eq/g plasma, in a third 150 $\mu$eq/g.

*Tissues.* The table summarizes the results obtained. It must be pointed out that Cl was determined on pieces taken from the same region as the samples used for Na and K but there may have been some variation in composition even in this restricted volume. The sodium space of the muscle and the chloride space of the electric tissue have been calculated and from these, together with the dry weight of the sample the internal K concentration has been estimated. The muscle contains more chloride than sodium, and the electric tissue more sodium than chloride.

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[1] Present address: Department of Biophysics, University College London.

| TISSUE | Na | K | Cl | DRY WEIGHT | K INTERNAL | Na OR Cl SPACE |
|---|---|---|---|---|---|---|
| | | *µeq/g* | | % | *µeq/g* | % |
| | | *First specimen* | | | | |
| Muscle | 21.5 | 98.5 | 24.7 | 22.4 | 151 | 12.3 |
| | 23.0 | 94.5 | . . | 21.8 | 146 | 12.6 |
| Electric organ (main) | 91 | 59.4 | 65 | 7.5 | 130 | 46.7 |
| Electric organ (Sachs) | 148 | 23.0 | 107 | 5.7 | 132 | 76.9 |
| | | *Second specimen* | | | | |
| Electric organ (main) | 99 | 54.6 | 73 | 6.9 | 135 | 52.6 |
| | 102 | 56.5 | . . | 5.6 | 135 | . . |
| Electric organ (Sachs) | 155 | 40.3 | 97 | 5.1 | 118 | 60.5 |
| | 145 | 44.5 | . . | 4.8 | 129.5 | . . |

*Red cells*

A sample taken after 20 min. centrifugation at 1500 g had the following analysis: Na 24, K 97.5, Cl 69.5 µeq/g cells and dry weight 30.8%.

## DISCUSSION

The electrolyte composition of the muscle of the electric eel resembles closely that of other muscles. A few measurements of the muscle membrane potential made in this laboratory showed values of 80–90 mV (Martins-Ferreira and Harris, unpublished). This range is similar to that found for the muscles of other species, and for the electroplates of the eel itself (Keynes and Martins-Ferreira, '53).

The electric organ is distinguished by the greater extracellular space it has in the form of layers between the rows of electroplates, this becomes still greater in Sachs' organ, corresponding to the greater separation of the electroplates. To account for the ratio of Na to Cl it seems necessary to suppose either (a) that some Na is intracellular or (b) that an organic anion replaces Cl in the extracellular space. The latter is preferable because in Sachs' organ the excess Na is so great that if it were present in the electroplates the magnitude of the action potential would be insufficient to give a reversal of sign of the potential difference between inside and outside, which is in fact observed.

The ratio of internal to external K concentration corresponds to a Donnan potential of about 90 mV. As this value is not far from the membrane potentials observed in the electroplates it appears that the K distribution may arise purely as the result of the operation of physical forces. For the potential to approximate that required for the Donnan equilibrium it is necessary to suppose further that in the resting state the permeability to Na is very low relative to that for K ions, for any flow of Na will lead to a reduced potential.

The red cell specimen of the electric eel approximates in electrolyte composition to the red cells of most other species.

## LITERATURE CITED

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# JOURNAL OF CELLULAR AND COMPARATIVE PHYSIOLOGY

VOL. 45 JUNE 1955 No. 3

## CONTENTS

ALBERT SCHATZ AND R. RAM MOHAN. Oxidation of ammonia and urea (heterotrophic nitrification) by *Streptomyces nitrificans*. Two figures . . 331

PAUL O. FROMM AND ROBERT E. JOHNSON. The respiratory metabolism of frogs as related to season. Four figures . . . . . . . . . . . . . . . . . . . . . . . . . . 343

N. SONNENSCHEIN AND M. J. KOPAC. The phosphatase activities of the microsomal (submicroscopic particulate) fraction prepared in an electrolyte medium. Four figures . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 361

W. H. FREYGANG, JR. AND W. M. LANDAU. Some relations between resistivity and electrical activity in the cerebral cortex of the cat. Seven figures . . 377

G. FRAENKEL. Inhibitory effects of sugars on the growth of the mealworm, *Tenebrio molitor* L. . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 393

MYRON LEVINE. An enzymatic comparison of two interbreeding but genetically isolated varieties of *Paramecium aurelia* . . . . . . . . . . . . . . . . . . . 409

THEODOR VON BRAND, EUGENE C. WEINBACH AND ELEANOR JOHNSON TOBIE. Comparative studies on the metabolism of the culture form and bloodstream form of *Trypanosoma gambiense* . . . . . . . . . . . . . . . . . . . . . . . 421

GEORGE G. BERG. Inorganic polyphosphatase in the developing frog. Eleven figures . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 435

A. S. V. BURGEN. The osmotic work of salivary secretion in the dog . . . . . . . 465

*Comments and Communications:*

EDWIN E. GORDON, MELVIN SPIEGEL AND CLAUDE A. VILLEE. An insulin effect on sponge metabolism. One figure . . . . . . . . . . . . . . . . . . . . . . . 479

KEICA NISHIE AND E. J. HARRIS. Electrolytes in the tissues of Electrophorus Electricus, L. . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . 484

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